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NUCLEAR IMPORT AND INTERACTIONS OF POTATO YELLOW DWARF VIRUS NUCLEOCAPSID, MATRIX, AND PHOSPHOPROTEIN

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NUCLEAR IMPORT AND INTERACTIONS OF
POTATO YELLOW DWARF VIRUS NUCLEOCAPSID,
MATRIX, AND PHOSPHOPROTEIN

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky

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2014

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ABSTRACT OF DISSERTATION

NUCLEAR IMPORT AND INTERACTIONS OF POTATO YELLOW DWARF VIRUS NUCLEOCAPSID, MATRIX, AND PHOSPHOPROTEIN

Potato yellow dwarf virus (PYDV) is the type species of the genus *Nucleorhabdovirus* and, like all members of this genus, replication and morphogenesis occurs inside the nuclei of infected cells. Protein localization prediction algorithms failed to identify a nuclear localization signal (NLS) in PYDV nucleocapsid (N) protein, although PYDV-N has been shown to localize exclusively to the nucleus when expressed as a green fluorescent protein (GFP):N fusion in plant cells. Deletion analysis and alanine-scanning mutagenesis identified two amino acid motifs, ⁴¹⁹QKR⁴²¹ and ⁴³²KR⁴³³, that were shown to be essential for nuclear import and interaction with importin- α . Additional bimolecular fluorescence complementation showed that the PYDV-N-NLS mutants cannot be ferried into the nucleus via interaction with PYDV-P or -M. In contrast, interaction with N-NLS mutants appeared to retard the nuclear import of PYDV-P. Taken together, it was determined that PYDV-N contains the bipartite NLS ⁴¹⁹QKRANEEAPPAAQKR⁴³³. Similarly, alanine-scanning mutagenesis was performed to determine the regions responsible for the nuclear import of PYDV-M and -P. A non-canonical NLS was identified in PYDV-P, consisting of three regions in the N-terminus of the protein required for nuclear import. PYDV-P does not interact with any *Nicotiana benthamiana* importins, but was found to interact with importin- α 7 and - α 9 of the non-host plant *Arabidopsis thaliana*. Two amino acids of PYDV-M, ²²⁵KR²²⁶, were found to be critical for nuclear import and interaction with importin- α . In addition, site-directed mutagenesis identified that amino acids ²²³LL²²⁴ of PYDV-M, which are adjacent to the two amino acids identified as responsible for nuclear import, are critical for inducing invaginations of the inner nuclear membrane. Bimolecular fluorescence complementation (BiFC) was then used to identify any differences in localization and interaction caused by the mutations introduced to PYDV-P and -M. The PYDV-P and -M proteins were still able to interact with other PYDV proteins, although the localization of the interaction differs between mutants.

KEYWORDS: rhabdovirus, nuclear import, nuclear localization signal, plant nucleus,
importin- α

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February 7th, 2014

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CHAPTER I

A review of nuclear import

A defining feature of all eukaryotes is the separation of genetic material from the cytoplasm by the double membrane of the nuclear envelope. Due to this partitioning, macromolecules such as proteins and nucleic acids must be imported and exported across the nuclear envelope. This is accomplished by selective transport across supramolecular structures that span the nuclear envelope called nuclear pore complexes (NPCs). Nuclear transport is critical for proper plant growth and development, and is also used by various viruses and the *Agrobacterium* genus of bacteria. This review will cover the various pathways and methods of nuclear import.

VIRAL NUCLEAR IMPORT STRATEGIES

Many viruses, including the majority of RNA viruses, never enter the nucleus and replicate in the cytoplasm of infected cells. Other viruses that need to enter the nucleus, such as retroviruses and papillomaviruses, overcome the barrier of the nuclear envelope by waiting until mitosis, when the nuclear envelope is disassembled. The remaining viruses that enter the nucleus are generally too large to pass through the NPC, and have developed various strategies to enter the nuclei of infected cells.

Members of the family *Herpesviridae* are large DNA viruses that replicate and package viral particles in the nucleus. The viruses lose their envelope upon entering infected cells. The viral capsid is then directed to the nucleus, where it docks with the NPCs (Ojala *et al.*, 2000). Similar to a bacteriophage, the capsid then undergoes

a conformational change, creating an opening in the capsid through which the viral DNA is released and transported through the NPC (Newcomb *et al.*, 2007).

Viruses in the family *Adenoviridae* also replicate in the nucleus. Similar to the herpesviruses, these viruses are uncoated in the infected cells, and the viral capsid is transported to the nucleus, where it docks with the NPC. However, instead of releasing the viral DNA into the pores, the viral capsid is disassembled at the cytoplasmic face of the NPC (Greber *et al.*, 1997). The viral DNA is then imported into the nucleus with the help of several cellular factors, including importins α and β , transportin, and importin 7 (Kobiler *et al.*, 2012).

The *Orthomyxoviridae* are enveloped negative-sense RNA viruses, whose most well known members are the influenza viruses. Unlike most RNA viruses, members of this family replicate inside the nucleus. The RNA segments of these viruses are coated with viral proteins, which form ribonucleoprotein complexes (RNPs). During infection, viral capsid is disassembled in the endosome, upon which the viral envelope fuses with the endosomal membrane, resulting in the release of the RNPs in the cytoplasm (Skehel and Wiley, 2000). It was found that the RNP proteins all contain functioning nuclear localization signals (NLSs) which are recognized by importin- α , however the NLS of the NP protein alone is sufficient to direct the nuclear import of the RNP (O'Neill *et al.*, 1995). Interestingly, it was found that the nuclear protein (NP) of mammalian influenza interacts with different isoforms of importin- α than avian influenza, which suggests that nuclear import may be responsible for determining host range (Gabriel *et al.*, 2011).

Members of the retrovirus subgroup *Lentivirus* enter host cells as a single-strand RNA viruses. Once in the cytoplasm of the host cell, the virus uses its own reverse transcriptase to generate DNA from its RNA genome, upon which the viral DNA is transported to the nucleus and incorporated into the host cell genome. As mentioned previously, most retroviruses wait until cell division and disassembly of the nuclear envelope to enter the nucleus. However, *Lentivirus* DNA associates with viral proteins in the cytoplasm, which transport the genomic DNA through the nuclear pores where it is integrated into the host genome (Kobiler *et al.*, 2012).

Hepadnaviruses are small, enveloped DNA viruses which have a single capsid. The capsid of human hepatitis B virus (HBV), for example, measures 34 nm in diameter (Crowther *et al.*, 1994). The capsid proteins of hepadnaviruses contain an NLS which can be exposed by phosphorylation (Rabe *et al.*, 2003). The exposed capsid NLS interacts with importin- α and β , and the intact capsids are imported through the NPC, but cannot pass the nuclear basket (Kann *et al.*, 1999). The mature, DNA-containing capsids then disassemble in the nuclear basket, leading to release of the viral genome into the nucleus (Rabe *et al.*, 2009).

The *Parvoviridae* viruses contain a single-stranded DNA genome encapsidated by a capsid that is only approximately 26 nm in diameter (Parrish and Berns, 2007). Using electron microscopy, these viruses have been observed to enter the nucleus as intact virus particles (Sonntag *et al.*, 2006). Two different methods have been proposed for import of these virus capsids. Four putative NLSs have been identified in a capsid protein of paroviruses which, due to the small size of the

capsid, suggests that the intact capsids may be imported into the nucleus via classical nuclear import (Lombardo, *et al.*, 2002). Alternatively, it has been observed that the parvovirus Minute virus of mice (MVM) causes disruptions of the outer nuclear membrane (ONM), which could lead to viral entry through the nuclear envelope (NE; Cohen *et al.*, 2006).

The different strategy for nuclear import is used by members of the *Polyomaviridae* family, which are double stranded DNA viruses. A notable member of this family is Simian virus 40 (SV40), whose large T antigen contains the first NLS to be characterized (Kalderon *et al.*, 1984). Disassembly of these viruses occurs in the ER of infected cells. It has been shown that two of the capsid proteins of SV40 are able to integrate into the ER, creating disruptions through which the disassembled viral particle can exit (Daniels *et al.*, 2006). Since the capsid proteins contain NLSs, it is proposed that the viral genome exits the ER into the cytoplasm, then enters through the NPC accompanied by the capsid proteins (Nakanishi *et al.*, 2002). Additionally, since the ER and the ONM are contiguous, the capsid proteins of these viruses may be able to disrupt the inner nuclear membrane (INM), allowing direct entry of the disassembled viral particle into the nucleus (Daniels *et al.*, 2006).

POTATO YELLOW DWARF VIRUS

The rhabdoviruses are easily identified by electron microscopy in infected cells by their distinctive enveloped bacilliform or bullet-shaped particles. As such, many plant rhabdoviruses, such as *Potato yellow dwarf virus* (PYDV) were identified based on electron microscopy of infected plants (Black *et al.*, 1948). In addition to the distinctive particle shape, more detailed analysis of these viruses identified

additional common properties. All members of the *Rhabdoviridae* are monopartite, negative-sense, single-stranded RNA viruses, with a consensus gene sequence flanked by leader (*l*) and trailer (*t*) regions of 3'-*l*-N-P-M-G-L-*t*-5'. All sequenced plant rhabdoviruses contain an additional one to four genes, some of which are likely involved in cell-to-cell movement in plants. In the case of PYDV, there are two additional genes, in the order of 3'-*l*-N-**X**-P-**Y**-M-G-L-*t*-5' (Bandyopadhyay *et al.*, 2010). The plant-adapted rhabdoviruses can be divided into two genera based on their sites of replication in infected cells: the genera *Cytorhabdovirus*, which replicate in the cytoplasm, and *Nucleorhabdovirus*, which replicate in host cell nuclei.

The rhabdovirus particles are easily distorted, and can vary in length (130-350 nm) and width (45-100 nm; Jackson *et al.*, 2005). Electron microscopy studies have shown average size of PYDV particles to be 380 nm in length and 75 nm in width (MacLeod *et al.*, 1966). Although only limited biochemical analyses have been conducted on plant rhabdoviruses, the probable function of the viral proteins can be deduced based on sequence-derived structure and similarity to animal rhabdoviruses (Jackson *et al.*, 2005). Despite the variance in particle size, all consist of a tightly coiled nucleocapsid core containing the viral genomic RNA, nucleocapsid protein (N), phosphoprotein (P), and polymerase protein (L). In addition to being a part of the minimal infectious unit, the N protein encapsidates the viral RNA, and is part of the polymerase complex (Wagner *et al.*, 1996). The P protein is also part of the minimal infectious unit, and may have several functions including transcription and replicase recycling, and suppression of host anti-viral defenses (Wagner *et al.*,

1997; Martins *et al.*, 1998; Jackson *et al.*, 2005). The last component of the minimal infectious unit is the L protein, which contains the polymerase and RNA-binding domains required for viral transcription (Jackson *et al.*, 2005). During morphogenesis, the matrix protein (M) condenses the nucleocapsid core, and interacts with glycoprotein spikes, formed by the viral G protein, that are embedded in a host membrane. Evidence suggests the function of PYDV-Y may be similar to the expected function of SYN-SC4, which has been implicated in cell-to-cell movement (Scholthof *et al.*, 1994; Goodin *et al.*, 2002; Min *et al.*, 2010). The function of PYDV-X is currently unknown.

Although the sequence and structure of several plant rhabdoviruses have been determined, little progress has been made in understanding the infection process of these viruses (Jackson *et al.*, 1987; Jackson *et al.*, 2005). Based on studies of PYDV, *Sonchus yellow net virus* (SYNV), and *Vesicular stomatitis virus* (VSV; Himangi *et al.*, 2004), Jackson *et al.* (2005) have proposed a model for the lifecycle of plant rhabdoviruses (Figure 1.1). Firstly, the virus enters the cytoplasm of a host cell via insect vectors or wounding. Current electron microscopy evidence suggests that the enveloped viral particle then associates with the endoplasmic reticulum, upon which the nucleocapsid core is released into the cytoplasm. Cytorhabdoviruses would then immediately begin transcription of viral mRNAs. The viral cores of nucleorhabdoviruses, such as PYDV, are then imported into the nucleus where transcription of viral mRNAs occurs (Jackson *et al.*, 2005). It is likely that PYDV follows a pathway similar to that of the negative-sense single-stranded RNA orthomyxoviruses, whose ribonucleoprotein are released in the cytoplasm before

being transported to the nucleus as a unit (O'Neill *et al.*, 1995). The viral mRNAs are then exported to the cytoplasm and translated, followed by transport of the viral proteins back into the nucleus for assembly of viral particles or cell-to-cell movement complexes. Viral particles are assembled on the inner nuclear membrane (INM), then bud through the INM embedded with glycoprotein (G) and accumulate in the peri-nuclear space. Additionally, there is evidence in a related nucleorhabdovirus (SYNV) of a cell-to-cell movement complex consisting of viral genomic RNA, nucleocapsid protein, and matrix protein that is exported from the nucleus where it associates with SYN-V and is directed to plasmodesmata (Min *et al.*, 2010).

Plant rhabdoviruses, such as PYDV, do not spread through seed or pollen transmission, and mechanical transmission is not expected to be a major pathway. Instead, these viruses are transmitted by insect vectors. Insect vectors, such as aphids and leafhoppers, acquire the virus by feeding on infected plant tissue. There is significant evidence that replication of plant rhabdoviruses also occurs in the insect vectors. There is a long latent period between uptake of the virus by the vector and transmission to subsequent plants, the virus is often retained throughout the life of the insect, and the virus can be transmitted through insect eggs (Nault, 1997; Sylvester and Richardson, 1992; Black, 1970). After acquiring the virus through feeding, the viral particles enter the insect midgut epithelial cells via receptor-mediated endocytosis. Evidence for this pathway comes from greatly reduced infectivity in insects when the viral glycoprotein is blocked by antibodies or enzymatically removed (Gaedigk *et al.*, 1986). The virus then spreads into the

haemolymph, then to the salivary glands, and ultimately into the insect saliva, allowing for infection of additional host plants.

NUCLEAR PORE COMPLEXES AND NUCLEOPORINS

The nucleoplasm of eukaryotic cells is separated from the cytoplasm by the double membrane of the nuclear envelope (NE). The NE consists of the inner nuclear membrane (INM), 30-55 nm of perinuclear space between membranes, and the outer nuclear membrane (ONM), which is contiguous with the ER. The only channel through which substrates can cross the nuclear envelope is the large protein complex known as nuclear pore complex (NPC; Figure 1.2). Nuclear pores were first identified more than 60 years ago in the nuclei of amphibian oocytes (Callan and Tomlin, 1950). Seventeen years after the discovery of NPCs in animals, Yoo and Bayley (1967) reported that nuclear pores in pea plants resemble those described in various animal cells. The general structure of NPCs is composed of eight symmetrical spokes, each consisting of a cytoplasmic and nuclear subunit, which are anchored between the INM and ONM forming an eightfold symmetrical pore (Suntharalingam *et al.*, 2003). Eight protein filaments extend from the cytoplasmic ring towards the cytoplasm, and eight protein filaments extend from the nuclear ring which converge to a ring-like structure named the nuclear basket (Suntharalingam *et al.*, 2003).

It has been shown by electron microscopy studies that this general NPC structure is conserved, even between distantly related species (Adam, 2001). Although the general NPC structure is conserved, the size of the pore complex and distribution varies between different organisms. Early electron microscopy studies

of vertebrates and higher eukaryotes estimated that NPCs have a mass of approximately 125 MDa (Reichelt *et al.*, 1990), whereas in yeast, NPCs are only approximately 66 MDa (Rout and Blobel, 1993). However, more recent estimates based on nucleoporin composition of the NPC suggest the mass is closer to 60 MDa in vertebrates, and 44 MDa in yeast (Cronshaw *et al.*, 2002; Rout *et al.*, 2000). There are far fewer reports regarding the mass and composition of plant NPCs (Heese-Peck and Raikhel, 1998), as a protocol for the biochemical isolation of plant NPCs has not been developed (Tamura and Hara-Nishimura, 2012). However, a study by Roberts and Northcote (1970) determined that plant NPCs are approximately 1150 x 640 Å, making them larger than those of yeast (960 x 350 Å), but smaller than the NPCs in vertebrates (1450 x 800 Å).

The distribution of NPCs also varies greatly between organisms. A single nucleus in vertebrates contains an average of 2,000-5,000 NPCs (10-20 pores μm^{-2}), however, yeast have only around 200 NPCs per nucleus (12 pores μm^{-2}), and *Xenopus laevis* nuclei can contain 5×10^7 NPCs (60 pores μm^{-2} ; Maul, 1977; Reichelt *et al.*, 1990; Rout and Blobel, 1993; Winey *et al.*, 1997). Roberts and Northcote (1970) estimated that plant nuclei contain approximately 64 pores μm^{-2} , which accounts for up to 38% of the nuclear envelope area. High-resolution scanning electron microscopy in tobacco cells clearly demonstrated that NPCs are non-randomly distributed over the nuclear envelope, which is consistent with other higher eukaryotes (Fiserova *et al.*, 2009). In addition, the NPC composition can also vary between cell types, cell cycle stage, and developmental stage within the same

organism. Metabolically active cells were observed to have NPCs with a larger inner diameter, and the NPCs of senescent cells had smaller inner diameters. (Fiserova *et al.*, 2009). These changes were also observed in *Saccharomyces cerevisiae*, vertebrates (Capelson and Hetzer, 2009), *X. laevis* (Goldberg *et al.*, 1997), and *Drosophila melanogaster* (Kiseleva *et al.*, 2001), which further suggests conservation of nuclear pore structure and functions across eukaryotes.

NUCLEOPORINS

Early estimates of NPC composition suggested these large complexes are made up of 50-100 proteins. However, a proteomics study by Rout *et al.* (2000) determined that *S. cerevisiae* NPCs are composed of only 29 different proteins, which have been collectively termed nucleoporins. A list of nucleoporins identified in plants, yeast, and vertebrates can be seen in Table 1.2. Two years after this study, vertebrate proteomics data identified 29 nucleoporins in rat liver nuclei (Cronshaw *et al.*, 2002). Obtaining knowledge of plant nucleoporins was slower than that of vertebrates, due to the inability to identify plant nucleoporins using homology-based approaches (Meier, 2006). However, an interactive proteomics study in 2010 by Tamura *et al.* identified 29 putative nucleoporins from *Arabidopsis*. Despite low primary sequence similarity, the overall shape and predicted folds of these nucleoporins were found to be broadly conserved among yeast, vertebrates, and plants (Neumann *et al.*, 2010). Based on these structural similarities, nucleoporins can be divided into five classes: transmembrane ring, core scaffold, cytoplasmic filaments, nuclear basket, and central FG nucleoporins (Tamura and Hara-Nishimura, 2013).

The transmembrane ring nucleoporins are responsible for anchoring the assembled NPC to the nuclear envelope. Two of these nucleoporins, Gp210 (Gerace *et al.*, 1982; Greber *et al.*, 1990) and NDC1 (Wozniak *et al.*, 1994), have been identified in plants, vertebrates, and yeast. Vertebrates and yeast each possess one additional transmembrane ring nucleoporin (Tamura and Hara-Nishimura, 2013). A gp210 knockout mutant of *Arabidopsis* was an embryo defective mutant, which suggests that this nucleoporin is essential in plant development (Meinke *et al.*, 2008).

The core scaffold nucleoporins connect the transmembrane ring nucleoporins to the central FG nucleoporins. The scaffold nucleoporins are the largest and most evolutionarily conserved class, consisting of 18 members in plants (Tamura and Hara-Nishimura, 2013). An outer ring is formed by 12 nucleoporins in plants, and is proposed to facilitate the smooth transition from the pore membrane to the inner and outer nuclear membranes (Alber *et al.*, 2007). In addition, electron microscopy and biochemical analysis of the outer ring complex in yeast determined that the structure contains two flexible hinge regions, which would allow a structural change in the NPC scaffold to allow large cargos to pass through (Kampmann and Blobel, 2009). Four nucleoporins form an inner ring (Tamura *et al.*, 2010), and two nucleoporins act as linkers between the core scaffold and the FG nucleoporins (Alber *et al.*, 2007).

The cytoplasmic filaments extend from the NPC into the cytoplasm, and the nuclear basket extends from the NPC into the nucleus. There are two nucleoporins

in plants and yeast that make up the cytoplasmic filaments. These filaments are predicted to play a role in specific interactions and serve as docking sites for nuclear import (Tamura and Hara-Nishimura, 2013). An additional cytoplasmic filament nucleoporin has been identified in vertebrates, but is absent in plants and yeast. This nucleoporin, Nup358, tethers RanGAP to the NPC (Hutten *et al.*, 2009), which plays an important role in facilitating directional nuclear import (discussed in further detail below). Nup358 is absent in plants, but all plant RanGAPs have an N-terminal domain called the WPP domain, which interacts with two nuclear envelope proteins (WIP and WIT) to anchor RanGAP to the NE (Xu *et al.*, 2007b, Zhao *et al.*, 2008).

The nuclear basket is comprised of three nucleoporins, which form eight filaments protruding into the nucleoplasm that converge into a ring-like structure (Strambio-De-Castillia *et al.*, 2010). This structure is expected to serve as a docking site for export of molecules out of the nucleus. In fact, two of these nucleoporins in *Arabidopsis* (Nup136 and NUA), have been shown to be involved in mRNA export (Xu *et al.*, 2007a; Tamura *et al.*, 2010). Additionally, *Xenopus* Nup153 (the Nup136 homologue) is the only nucleoporin that interacts with lamin B, which suggests that Nup136 in plants could be responsible for linking the NPC to the nuclear lamina (Tamura and Hara-Nishimura, 2013).

The central FG nucleoporins are named as such due to a characteristic structural motif consisting of multiple repeats of phenylalanine and glycine residues (Cronshaw *et al.*, 2002). These motifs result in nucleoporins with an unfolded

structure, allowing for multiple low-affinity and high-specificity interactions with transport factors (Strawn *et al.*, 2004). These nucleoporins, five of which have been identified in plants, are oriented perpendicular to the central channel, and form the selective barrier that mediates transport of proteins through the NPC (Tamura and Hara-Nishimura, 2013). Interestingly, all known FG nucleoporins have the ability to interact with at least one transport receptor (Ryan and Wente, 2000). Several models have been proposed to explain how the central FG nucleoporins mediate directionality of transport, and are discussed below.

PROPOSED MODELS FOR NPC SELECTIVITY

Although the structure and composition of the NPC is now better understood, the exact mechanism by which the nucleoporins confer selective transport still needs to be determined. Only macromolecules under approximately 40 kDa or 5 nm in diameter can freely diffuse through nuclear pores, and larger substrates up to 39 nm in diameter can be accommodated with the aid of transporters (Pante and Kann, 2002). It is known that the central FG repeat nucleoporins form the selective barrier in the central channel of NPCs (Tamura and Hara-Nishimura, 2013) and that all known FG nucleoporins have the ability to interact with at least one transport receptor (Ryan and Wente, 2000). Additionally, it has been shown that translocation through NPCs is dependant on interaction of transport receptors with nucleoporin FG motifs (Weis, 2003; Bednenko *et al.*, 2003a), and that removing the FG binding motif of importin- β prevents the import of cargos that use this receptor (Bednenko *et al.*, 2003b; Bayliss *et al.*, 2000). Based on this information, several models for selective transport through the NPC have been proposed.

One model, proposed by Rout *et al.* in 2000, suggests that movement across NPCs is due to Brownian motion. In this 'Brownian affinity-gating' model, passive diffusion of macromolecules that do not bind nucleoporins are inhibited by filamentous FG nucleoporins that surround the narrow central channel. However, macromolecules that do bind FG nucleoporins are retained near the entrance of the central channel, which increases diffusion across the NPC. Rapid and reversible binding to these FG nucleoporins promotes diffusion through the central channel. In this model, directionality of transport is due to the asymmetrical distribution of Ran-GTP in the nucleus. When the cargo/transporter complex diffuses across the pores, the transporter preferentially binds Ran-GTP, leading to the release of the cargo in the nucleoplasm. The physical barrier created by the FG nucleoporins around a central channel would explain why smaller molecules are able to diffuse across the NPC much easier than larger molecules. Additionally, in this model, the observed dilation of the NPC central channel during transport of large substrates would be a consequence, rather than cause, of substrate translocation (Rout *et al.*, 2000).

A second model, termed the 'selective-phase' model, was proposed in 2001 by Ribbeck and Gorlich. In this model the FG nucleoporins that line the central channel of the NPC interact to form a hydrophobic network (Ribbeck and Gorlich, 2001). This network acts as a mesh that only allows the passage of small molecules without the aid of a transporter. When larger molecules form complexes with transporters, the transporters interact with the FG repeats and cause the meshwork to be disrupted, allowing for passage through the NPC. Some support for this model

came from Frey *et al.* (2006), who showed that the FG motifs of a yeast nucleoporin, Nsp1p, form hydrogel-like structures *in vitro*. In addition this study found that substituting the phenylalanine in the FG motifs for serine abolished the formation of hydrogels, and was lethal in yeast (Frey *et al.*, 2006). The currently available data provides the most support for this model of NPC selectivity.

Along with the selective-phase model, another model for selective transport across the NPC was proposed in 2001 (Macara, 2001). As opposed to a meshwork, this model proposes that the FG nucleoporins fill the pore like loose, oily spaghetti, leaving a relatively open central channel. As such, this model was termed the 'oily spaghetti' model (Macara, 2001). In this model, the inner diameter of the NPC is approximately 9 nm, and is coated by a 7 nm-thick layer of freely-moving nucleoporin 'spaghetti'. They suggest that the nucleoporins can move freely at physiological temperatures, but would have reduced movement at lower temperatures, leading to cargo being trapped in the NPC central channel. This model would allow for the diffusion of small molecules across the NPC, but would block diffusion of larger substrates without the aid of a transporter. Similar to the Brownian affinity-gating model, this model proposes that substrate/transporter complexes move randomly throughout the central channel by transient association with the FG nucleoporins. Due to the flexibility of the FG nucleoporins at physiological temperatures, the substrate/transporter complexes could easily push the nucleoporins aside (Macara, 2001).

A fourth model for nuclear transport posits that importin- β binding affinity to FG nucleoporins increases from the cytoplasmic side to the nuclear side of the NPC (Ben-Efraim and Gerace, 2001). The authors proposed the directional transport of cargo from the cytoplasm to the nucleus is due to the increasing affinity of importin- β for nucleoporins on the nuclear side of the NPC. It was also suggested that import complexes could transfer between nucleoporins due to the presence of more than one FG interacting domain in importin- β (Ben-Efraim and Gerace, 2001). This model implies that directional transport is due to the inherent structure of the NPC. However, there are some issues with this model. The majority of FG nucleoporins are distributed symmetrically through the NPC, and the asymmetrically distributed nucleoporins are not required for most nuclear import pathways (Zeitler and Weis, 2004). Additionally, direction of nuclear transport can be reversed with high concentrations of Ran-GTP in the cytoplasm (Nachury and Weis, 1999), which suggests that directional transport is not an inherent property of NPCs.

MECHANISM OF NUCLEAR IMPORT

After the discovery of nuclear localization signals in SV40 large T antigen and nucleoplasmin, there are two likely mechanisms for how NLSs may result in transport across the NPC. Cargos containing NLSs could bind directly to the NPCs and be translocated through the pores, or the NLS-containing cargos could be recognized by transporters, which would then carry them through the pores (Macara, 2001). Evidence for the transporter model was obtained when Adam *et al.* (1990) discovered that nuclear transport in mammalian cells is dependent on

soluble cytoplasmic factors. It was later discovered that nuclear transport could be reconstituted by adding soluble transport factors, one of which - importin- α - could directly bind to a classical NLS-containing protein (Adam and Gerace, 1991). The other isolated soluble factors required to reconstitute nuclear import were Ran, NTF2, and importin- β (Adam and Gerace, 1991). These factors were determined to be essential for the classical nuclear import pathway, and led to a model for classical nuclear import (Gorlich and Kutay, 1999; Moore and Blobel, 1994; Paschal and Gerace, 1995).

The current model for classical import requires the interaction of an NLS-containing cargo protein and the receptor importin- α in the cytoplasm (Figure 1.3). This complex then interacts with importin- β (via the importin- β binding [IBB] domain in importin- α), and forms a trimeric import complex. Importin- β interacts with specific nucleoporins in the NPC, and facilitates translocation through the pore. On the nuclear side of the NPC, Ran-GTP binds to importin- β , which causes a conformational change in importin- β . This conformational change interferes with interaction of the IBB-domain of importin- α , dissociating the trimeric complex (Gilchrist *et al.*, 2002). The exportin CAS bound to Ran-GTP interacts with importin- α , leading to the dissociation of cargo and the export of CAS/Ran-GTP/importin- α back through the NPC and into the cytoplasm (Kutay *et al.*, 1997). It has also been reported that one of the nuclear basket nucleoporins, Nup50, displaces cargo proteins from importin- α in the nucleus (Matsuura and Stewart, 2005). Similarly,

the importin- β /Ran-GTP complex is exported back into the cytoplasm, where it is hydrolyzed to Ran-GDP, leading to the release of importin- β .

Nuclear import is a non-directional process of facilitated diffusion that does not directly require energy or motor proteins (Ribbeck *et al.*, 1999). However, this process is able to move molecules into or out of the nucleus against a concentration gradient. This is possible due to the asymmetrical distribution of the small GTPase Ran between the nucleus and the cytoplasm. The presence of a guanine nucleotide exchange factor (RanGEF) restricted to the nucleus results in a high nuclear concentration of Ran-GTP, whereas Ran-GDP is abundant in the cytoplasm due to the cytoplasmic GTPase-activating protein RanGAP. This gradient leads to directional transport, as importin binding to cargo and binding to Ran-GTP are mutually exclusive. Transportin1, for example, has a 10,000-fold higher affinity for Ran-GTP than Ran-GDP (Macara, 2001). This allows cargo to bind transportin in the cytoplasm, where Ran-GTP concentration is very low, and release the cargo in the nucleus, where Ran-GTP concentration is much higher. Similarly, the export factor CAS can only form a complex with cargo proteins in the presence of Ran-GTP (Kutay *et al.*, 1997). After export, Ran-GTP is hydrolyzed to Ran-GDP dissociating the complex. This means every round of transport results in the export of one Ran molecule to the cytoplasm. Ran is returned to the nucleus by the transport factor NTF2, which is one of the soluble factors discovered to be critical for nuclear import by Adam and Gerace in 1991. NTF2 is only able to recognize Ran-GDP, and is able to transport Ran-GDP through the NPC. RanGEF in the nucleus then disassembles the complex by converting Ran-GDP to Ran-GTP (Ribbeck *et al.*, 1998).

TRANSPORT RECEPTORS AND ADAPTOR PROTEINS

The majority of nuclear transport receptors are members of the karyopherin- β family of proteins, and these proteins are responsible for the import and export of all proteins larger than the diffusion limit of the NPC (Mosammaparast and Pemberton, 2004). At least 20 members of this family have been identified in humans, 14 in yeast, and 17 in *Arabidopsis thaliana* (Merkle, 2003; Mosammaparast and Pemberton, 2004). One common feature of all karyopherin- β family members is the ability to interact with at least one of the FG nucleoporins (Chook and Blobel, 2001; Macara, 2001), which line the central channel of the NPC. Another shared feature of this family is their ability to bind and form a complex with Ran-GTP (Chook and Blobel, 2001; Merkle, 2003), whose importance in the nuclear transport cycle has been previously discussed. Import or export mediated by karyopherin- β proteins is typically unidirectional, and regulated by the Ran-GTP/GDP gradient described above. However, two karyopherin- β members have been identified that may operate in both directions - importin 13 (Mingot *et al.*, 2001), and exportin 4 (Lipowsky *et al.*, 2000). In addition to binding cargos directly, some karyopherin- β family proteins can be linked to cargos via adapter proteins, which is discussed below. Of the karyopherin- β members identified in plants, seven have been functionally characterized. These are the importins importin- β (Jiang *et al.*, 1998), transportin 1 (Ziemienowicz *et al.*, 2003), SAD2 (Zhao *et al.*, 2007), and the exportins exportin 1/XPO1 (Haasen *et al.*, 1999), exportin 2/CAS (Haasen and Merkle, 2002), exportin-T (Hunter *et al.*, 2003), and exportin 5 (Bollman *et al.*, 2003).

Importin- β was the first nuclear transporter to be identified (Adam and Gerace, 1991), and is able to carry cargo either directly or through an adaptor protein. Importin- β is able to directly bind a wide range of cargo proteins, with little similarity in the structural modes of interaction (Marfori *et al.*, 2011). Additionally, the NLSs that confer interaction with importin- β vary widely, ranging from 9 amino acids for the HIV Tat protein (Truant and Cullen, 1999) to 120 amino acids for interaction with SREBP-2 (Nagoshi *et al.*, 1999). As opposed to NLSs recognized by importin- α , importin- β -NLS binding does not appear to have a conserved mechanism, demonstrated by the wide range of NLSs that directly bind to importin- β (Marfori *et al.*, 2011). In addition to direct cargo binding, importin- β can interact with several adaptors to greatly increase the range of cargos able to be imported. Some of these adaptors include importin- α , snurportin (Huber *et al.*, 1998), XRIP α (Jullien *et al.*, 1999), and importin-7 (Jakel *et al.*, 1999).

The import pathway mediated by transportin 1/karyopherin- β 2 has also been characterized. Similar to direct cargo binding of importin- β , a common NLS could not be determined by comparing the sequences of over 20 proteins known to be imported by transportin 1 (Marfori *et al.*, 2011). However, structural analysis of transportin 1/cargo binding led to the discovery of an NLS recognized by transportin 1, termed the PY-NLS (Lee *et al.*, 2006). This illustrates the difficulty in determining import receptors based on sequence analysis, as cargos containing this type of NLS may be functionally similar but have differing amino acid sequences. Similarly, transportin 1 and importin- β are functionally similar, but only share

~14% sequence identity (Macara, 2001). Transportin 1 is also responsible for nuclear import of cargos containing the well characterized, but non-canonical, M9 NLS (Pollard *et al.*, 1996).

Exportin 1 (Xpo1)/Crm1 is a member of the karyopherin- β family of proteins and was the first export carrier to be identified (Ossarehnazari *et al.*, 1997). Similar to the importins, export karyopherins recognize specific peptide signals, referred to as nuclear export signals (NES). Exportin 1 recognizes a leucine-rich, hydrophobic NES that has been identified in over 75 proteins and is conserved in all eukaryotes (Lacour *et al.*, 2003; Stade *et al.*, 1997). Like importin- β , exportin 1 is able to extend its range of cargos by interacting with adapter proteins. Two adaptors that have been identified for exportin 1 are PHAX (Ohno *et al.*, 2000) and NMD3, which is needed for the export of the 60S ribosomal subunit (Gadal *et al.*, 2001; Ho *et al.*, 2000).

THE ADAPTOR PROTEINS

The first adaptor protein to be identified as a necessary step for nuclear import is importin- α (Gorlich *et al.*, 1994). As mentioned previously, in the classical nuclear import pathways, importin- α binds a cargo protein and interacts with importin- β , forming a trimeric complex that moves through the NPC into the nucleus and dissociates. The function of importin- α is to act as a receptor for NLS-containing cargo proteins in the cytoplasm, thereby increasing the range of possible cargos of importin- β . Further increasing the range of possible cargos is the presence of different isoforms of importin- α within the same species. Humans and *A. thaliana*

have six and eight isoforms of importin- α , respectively, however only one importin- α is known in yeast (Kosugi *et al.*, 2009b). Importin- α is composed of two domains: the N-terminal importin- β binding (IBB) domain, and a C-terminal domain consisting of tandem armadillo (Arm) repeats (Marfori *et al.*, 2011). The stacked Arm repeats lead to the formation of two NLS-binding pockets in importin- α : a 'major' binding groove and a 'minor' binding groove (Conti and Kuriyan, 2000). A study of consensus classical NLS sequences by Kosugi *et al.* (2009b), determined that cargos binding importin- α can be divided into six classes based on whether they interact with the major groove, minor groove, or both.

There are some interesting differences between the importin- α members in plants compared to animals. Firstly, importin- α in animal cells is mostly cytosolic, and can be easily removed from permeabilized cells. However, in plants, importin- α has a very tight association with the nuclear envelope, even in permeabilized cells (Hicks *et al.*, 1996). Secondly, in animal and yeast cells, importin- β is necessary for formation of the trimeric import complex and required for high affinity binding between importin- α and an NLS-containing protein. This is due to an autoinhibitory function of mammalian importin- α when the minor NLS binding groove interacts with the IBB-domain (Harreman *et al.*, 2003). Conversely, plant importin- α is able to bind with high affinity to cargo proteins even in the absence of importin- β (Hubner *et al.*, 1999). It has also been observed in plant cells that importin- α can associate with microtubules, but this association only occurs in the presence of an NLS-containing cargo protein (Smith and Raikhel, 1999).

Another example of a well-characterized importin- β adaptor is snurportin-1. Uridine-rich small nuclear ribonucleoprotein particles (U snRNPs) contain import signals on both the m₃G cap on the RNA of the U snRNP and sequences within the core protein of the RNP that are recognized by the adaptor snurportin-1 (Huber *et al.*, 1998; Palacios *et al.*, 1997). Snurportin-1 contains an IBB domain with a high degree of similarity to importin- α , and binds importin- β by a similar mechanism (Mitrousis *et al.*, 2008).

Two other adaptor proteins, XRIP α and importin-7, also transport cargos into the nucleus in a complex with importin- β , and are not dependant on the presence of importin- α . XRIP α is responsible for import of RPA in *Xenopus*, and interacts with importin- β through a specific N-terminal binding sequence, similar to that of importin- α or snurportin (Jullien *et al.*, 1999). Importin-7 has been shown to facilitate the nuclear import of histone H1 by binding with both H1 and importin- β (Jakel *et al.*, 1999).

NUCLEAR LOCALIZATION SIGNALS

The first evidence that specific peptide signals could be responsible for the nuclear import of proteins came from studying the nuclear chaperone protein nucleoplasmin. It was found that proteolytic hydrolysis of the C-terminal amino acids of nucleoplasmin inhibited its import to the nucleus (Dingwall *et al.*, 1982). It was also observed that the C-terminal 50 amino acids of nucleoplasmin were able to accumulate in the nucleus of *Xenopus laevis* oocytes (Dingwall *et al.*, 1982), which suggested that nuclear import is a selective process, and can be mediated by a

peptide signal present in the carboxy terminus of nucleoplasmin. Despite the findings of Dingwall *et al.* (1982), the first nuclear localization signal was not identified for another two years. In 1984, Kalderon *et al.* reported the discovery of a nuclear localization signal (NLS) in the SV40 large T antigen. The sequence was determined to consist of a basic region of seven amino acids, namely PKKKRKV, and was demonstrated to be necessary for the nuclear import of SV40 large T antigen, and sufficient to promote the nuclear localization of unrelated proteins (Kalderon *et al.*, 1984). Several years later, Dingwall *et al.* (1988) determined the specific amino acid sequence responsible for the nuclear import of nucleoplasmin. This sequence, KRPAATKKAGQAKKKKLD, consists of two basic regions of amino acids (underlined), both of which are essential for nuclear import (Dingwall *et al.*, 1988). These two NLSs are now considered to be prototypes for nuclear import, and has led to 'classical' NLSs being defined as consisting of a single or bipartite sequence rich in basic amino acids.

A bioinformatic analysis of *S. cerevisiae* determined that 57% of proteins with known nuclear localization contain a classical NLS (31% monopartite, 26% bipartite; Lange *et al.*, 2007). However, a more recent review using multiple prediction algorithms suggests this number may be closer to 30% in yeast, and 40% in mice (Marfori *et al.*, 2011). It has previously been reported that the prediction algorithms used by Marfori *et al.* are more sensitive than those used by Lange *et al.* (Kosugi *et al.*, 2009a), which supports the revised estimates. This suggests that up to 70% of known *S. cerevisiae* nuclear-localized proteins contain non-classical nuclear localization signals. However, there is some evidence that the classical

import pathway may be the most important. For example, the only known function of exportin-2 to date is to recycle importin- α back to the cytoplasm for another cycle of nuclear import (Haasen and Merkle, 2002; Kutay *et al.*, 1997).

Although the classical NLSs were the first to be identified and may be the most common, many different classes of NLSs have now been identified. Kosugi *et al.* (2009b) divided classical importin- α -binding NLSs into six classes based on sequence similarities and their mode of interaction with importin- α . As discussed in more detail later, importin- α consists of two NLS-binding grooves, the 'major' binding groove and the 'minor' binding groove. Class 1 and 2 NLSs both interact with the major groove of importin- α . Class 1 NLSs are small, monopartite, and rich in basic amino acids with a consensus sequence of KR[KR]R or K[KR]RK (square brackets indicate either residue may be at this position; Kosugi *et al.*, 2009b). The SV40 NLS (PKKKRKV) is an example of a Class 1 NLS. Class 2 NLSs also bind the major groove of importin- α and consist of a basic core, but the import activity varies depending on the flanking residues. Class 2 NLSs include the c-Myc NLS (PAAKRVKLD), and have a consensus sequence of [PR]X₂KR{DE}[KR] (X indicates any amino acid may be used at this position; braces indicate any amino acid other than D or E may be used in this position; Kosugi *et al.*, 2009b). Additionally, the basic residues in Class 2 NLSs could be substituted for hydrophobic residues while still maintaining import activity.

NLSs of the Class 3 or Class 4 subgroups specifically bind to the minor groove of importin- α . Class 3 and 4 NLSs differ from Class 1 or 2, in that they only contain

two or three basic amino acids. The consensus sequences for Class 3 and Class 4 NLSs were determined to be $KRX[WFY]X_2AF$ and $[RP]X_2KR[KR]\{DE\}$, respectively (Kosugi *et al.*, 2009b). While NLSs of Classes 1-4 were shown to interact with most importin- α isoforms from yeast, rice, and humans, NLSs of Class 5 (consensus $LGKR[KR][WFY]$) strongly interacted with only rice importin- α , which suggests this class of NLSs is specific to plants (Kosugi *et al.*, 2009b). However, a more recent study showed that two proteins containing a Class 5 NLS were able to bind yeast, human, and plant importin- α isoforms, although they showed a preference for plant importin- α (Chang, *et al.*, 2012).

The final class of importin- α -binding NLSs, Class 6, consists of two basic regions of amino acids separated by a linker region. These bipartite NLSs, such as the Nucleoplasmin NLS, interact with both the major and minor grooves of importin- α . The linker region can be between 10 and 20 residues and can consist of any amino acids, however, import activity is increased by acidic residues in the central linker region. The consensus sequence for bipartite NLSs was determined to be $KRX_{10-12}K[KR][KR]$ or $KRX_{10-12}K[KR]X[KR]$ (Kosugi *et al.*, 2009b).

Atypical nuclear localization signals

Soon after the discovery of the classical nuclear import pathway, it became clear that many nuclear-localized proteins do not contain classical NLSs, and may use alternate pathways to enter the nucleus. One of the first non-classical NLSs to be discovered was the Mat $\alpha 2$ NLS in yeast (Hall *et al.*, 1984). The minimal sequence required for nuclear localization in *S. cerevisiae* Mat $\alpha 2$ was determined to

be a short sequence of both basic and hydrophobic amino acids, KIPK (Hall *et al.*, 1984). Although most NLSs are functional across humans, plants, and yeast, the Mat $\alpha 2$ NLS is not functional in mammals (Chelsky *et al.*, 1989). Later studies showed that the Mat $\alpha 2$ NLS is functional in plants *in vivo*, and that a *Zea mays* transcription factor contains a Mat $\alpha 2$ -like NLS, RKAIGKR (Hicks *et al.*, 1995).

Another atypical NLS was identified in the C-terminus of the heterogeneous nuclear RNP (hnRNP) A1 protein which, unlike previously discovered NLSs, did not contain a core of basic residues. Nuclear import of this protein was found to be due to a 38 amino acid NLS (NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY), termed M9, which was somewhat conserved between hnRNP proteins in humans and *X. laevis* (Siomi and Dreyfuss, 1995). It was later found that nuclear import of the M9 NLS is mediated by transportin 1 (Pollard *et al.*, 1996), and occurs independently of importin- α (Michael *et al.*, 1995). Interestingly, it was also found that the M9 sequence is also responsible for the nuclear export of hnRNP A1, and that the NLS and NES functions cannot be uncoupled (Michael *et al.*, 1995). A rough consensus for M9-type NLSs that interact with transportin 1 was determined to be [YFW]X₂XSXZG[PK][MLV][KR], where J indicates a hydrophilic residue, and Z indicates a hydrophobic residue (Bogerd *et al.*, 1999).

A third group of atypical NLSs was identified not by sequence similarity, but by structural analysis of cargo/transportin 1 interaction. It was found that proteins that interacted with transportin 1 did so at three sites: an N-terminal motif, a central arginine-residue, and a C-terminal PY sequence (Marfori *et al.*, 2011). Three rules

for the PY-NLS were determined: they are structurally disordered in free cargos, they have an overall basic character, and they have a central hydrophobic or basic motif followed by the consensus sequence [RHK]X₂₋₅PY (Marfori *et al.*, 2011). Based on these rules, the PY-NLS has been identified in over 100 proteins, and has been experimentally validated for several (Lee *et al.*, 2006).

The NLS sequences that directly bind importin- β appear to be highly variable, but generally have a basic character. The NLS of human parathyroid hormone-related protein (PTHrP) directly binds to importin- β and is generally basic, but is much more complex than the classical NLSs. This NLS, consisting of 29 amino acids (See Table 1.1) was shown to be sufficient for nuclear import in the absence of importin- α (Lam *et al.*, 1999). Similarly, the sterol regulatory element binding protein 2 (SREBP-2) directly binds importin- β and contains a large 61 amino acid NLS with basic character (See Table 1.1; Nagoshi and Yoneda, 2001). The interaction of the SREBP-2 NLS with importin- β differs from that of the PTHrP NLS, as importin- β must adopt a more twisted and open conformation to accommodate SREBP-2 binding (Lee *et al.*, 2003). Another complex, basic NLS has been identified in the ribosomal protein L23a. This 42 amino acid sequence, designated BIB (Table 1.1), can be recognized by multiple carriers, including importin- β and transportin (Jakel and Gorlich, 1998).

Several NLSs that directly bind importin- β have also been identified in viral proteins, such as human T-cell leukemia virus type 1 (HTLV-1) Rex protein (MPKTRRRPRRSQRKPPT; Palmeri and Malim, 1999) and human

immunodeficiency virus (HIV) Rev and Tat proteins (RQARRNRRRRWR and RKKRRQRRR, respectively; Truant and Cullen, 1999). Based on these examples, a consensus sequence for viral NLSs was determined to be $RX_2RRX_{1-2}RBR$, where B indicates any basic residue (Macara, 2001).

Although the mechanism of interaction with importin- α has been well characterized, there are still non-classical NLSs whose nuclear import is mediated by importin- α . For example, the phospholipid scramblase 1 (PLSCR1) protein binds the major groove of importin- α , but contains a short, hydrophobic NLS (GKISKHW; Chen *et al.*, 2005) that does not fit into either of the importin- α major groove-binding classes from Kosugi *et al.* (2009b). Additionally, the Borna disease virus p10 protein contains a non-classical NLS that interacts with importin- α (RLTLLELVRRRLNGN; Wolff *et al.*, 2002).

Another interesting mechanism has been observed in the STAT (signal transducers and activators of transcription) proteins. STAT1 and STAT2 have been shown to interact with importin- α , but do not contain classical NLSs. It is known that STAT1 forms homodimers and heterodimers with STAT2. An element was identified in the STAT proteins (KEQK) that was not able to mediate nuclear import on its own. However, the formation of STAT1 homodimers or STAT1/STAT2 heterodimers allows the KEQK elements from each subunit to constitute a functioning NLS, which resembles a classical NLS and was able to interact with importin- α (Fagerlund *et al.*, 2002; McBride *et al.*, 2002; Melen *et al.*, 2001).

Along with NPCs and transport molecules, NLSs appear to be functionally conserved across kingdoms. For example, the classical SV40 NLS identified in animal cells also functions in plants and yeast (Lassner *et al.*, 1991). Additionally, the bipartite NLS identified in *Agrobacterium* VirD2 protein functions in plant, *Xenopus*, *Drosophila*, mammalian, and yeast cells (Guralnick *et al.*, 1996; Relic *et al.*, 1998). However, not all NLSs are conserved and functional across all kingdoms. The Mat $\alpha 2$ NLS, which was identified in yeast also functions in plant cells, but is not functional in animal cells (Chelsky *et al.*, 1989). Another interesting exception is the *Agrobacterium* VirE2 protein which contains two bipartite NLSs functional in plants, but not in any other non-plant organisms. However, by making it conform to the animal bipartite NLS consensus with a single amino acid mutation, it becomes functional in *Xenopus* and *Drosophila* (Guralnick *et al.*, 1996; Hicks, 2002).

CONCLUDING REMARKS

The above examples illustrate the difficulty in predicting nuclear localization based on sequence analysis. It is estimated that 60-70% of all nuclear-localized proteins do not contain classical NLSs (Marfori *et al.*, 2011), which means the specific NLS sequence must be experimentally verified in most cases. Table 1.1 demonstrates the diversity of currently identified types of NLS. In addition, the amino acid sequences of NLSs are not sufficient to determine the specificity for transport receptors (Friedrich *et al.*, 2006). Further complicating localization prediction is that NLSs, unlike most signals for organelle targeting, are not removed following import, allowing the protein to shuttle between the nucleus and cytoplasm. Cargo proteins can also contain more than one NLS, and multiple NLSs

can act cooperatively to increase import to the nucleus (Hicks and Raikhel, 1995). Lastly, there is even evidence that some proteins do not contain NLSs that interact with transporters or adaptors, but instead are imported by interaction with proteins containing functional NLSs (Leslie *et al.*, 2004; Shiota *et al.*, 1999; Xia *et al.*, 1992). Although much progress has been made in understanding the various nuclear import pathways, the above reasons show that there is much more to discover to fully understand and accurately predict the localization of proteins and methods of transport into the nucleus.

TABLE 1.1. Classes of Nuclear Localization Signals

NLS Type	NLS Class	Consensus sequence	Reference
Classical importin- α binding	Class 1	KR[KR]R or K[KR]RK	Kosugi <i>et al.</i> , 2009b
	Class 2	[PR]X ₂ KR{DE}[KR]	
	Class 3	KRX[WFY]X ₂ AF	
	Class 4	[RP]X ₂ KR[KR]{DE}	
	Class 5	LGKR[KR][WFY]	
	Class 6	KRX ₁₀₋₁₂ K[KR][KR] or KRX ₁₀₋₁₂ K[KR]X[KR]	
Non-classical importin- α binding	PLSCR1	GKISKHW	Chen <i>et al.</i> , 2005
	BDV p10	RLTLLELVRRNLGN	Wolff <i>et al.</i> , 2002
	STAT1	KEQK/KEQK	Melen <i>et al.</i> , 2001
	Mat α 2-like	KIPIK or RKAIGKR	Hall <i>et al.</i> , 1984; Hicks <i>et al.</i> , 1995
Transportin-binding	M9-like	[YFW]X ₂ XSXZG[PK][MLV][KR]	Bogerd <i>et al.</i> , 1999
	PY-NLS	[RHK]X ₂₋₅ PY	Marfori <i>et al.</i> , 2011
Importin- β binding	Viral-like	RX ₂ RRX ₁₋₂ RBR	Macara, 2001
	PTHrP	RYLTQETNKKVETYKEQPLKTPG KKKKGKP	Lam <i>et al.</i> , 1999
	SREBP-2	RSSINDKIIELKDLVMGTDAM HKSGVLRKAIDYIKYLQQVNHK LRQENMVLKLANQKNKL	Nagoshi and Yoneda, 2001
	BIB	VHSHKKKKIRTSPFTTPKTLR LRRQPKYPRKSAPRRNKLDHY	Jakel and Gorlich, 1998

TABLE 1.2. Identified nucleoporins in plants, yeast, and vertebrates

Nucleoporin Group	Plants	Yeast	Vertebrates	Reference
Cytoplasmic FG-Nups and filaments	Nup214	Nup159	Nup214	[1] [2]
	CG1	Nup42	CG1	
	-	-	Nup358	
Outer ring Nups	Nup160	Nup120	Nup160	[1] [3] [4]
	Nup133	Nup133	Nup133	
	Nup107	Nup85	Nup107	
	Nup96	Nup145C	Nup96	
	Nup75	Nup85	Nup75	
	SEH1	SEH1	SEH1	
	SEC13	SEC13	SEC13	
	Nup43	-	Nup43	
	Elys	-	Elys	
	Aladin	-	Aladin	
	GLE1	-	GLE1	
	RAE1	-	RAE1	
	-	-	Nup37	
Central FG-Nups	Nup98	Nup145N	Nup98	[1] [5] [6]
	Nup62	Nsp1	Nup62	
	Nup58	Nup49	Nup58/45	
	Nup54	Nup57	Nup54	
	Nup35	Nup53	Nup35	
	-	Nup116	-	
	-	Nup100	-	
	-	Nup59	-	
Inner ring Nups	Nup205	Nup192	Nup205	[7]
	Nup188	Nup188	Nup188	
	Nup155	Nup157/170	Nup155	
	Nup35	Nup53	Nup35	
	-	Nup59	-	
Transmembrane ring Nups	Gp210	Pom152	Gp210	[8] [9] [10]
	NDC1	NDC1	NDC1	
	-	Pom34	-	
	-	-	Pom121	
Linker Nups	Nup93	Nic96	Nup93	[1]
	Nup88	Nup82	Nup88	[3]
Nuclear FG-Nups and basket	Nup1/136	Nup1/60	Nup153	[1]
	Nup50	Nup2	Nup50	[7]
	NUA	Mlp1/2	Tpr	[11]

[1] Tamura and Hara-Nishimura, 2013 [2] Hutten *et al.*, 2009 [3] Alber *et al.*, 2007 [4] Kampmann and Blobel, 2009
[5] Cronshaw *et al.*, 2002 [6] Ryan and Wentle, 2000 [7] Tamura *et al.*, 2010 [8] Gerace *et al.*, 1982 [9] Greber *et al.*, 1990
[10] Wozniak *et al.*, 1994 [11] Xu *et al.*, 2007a

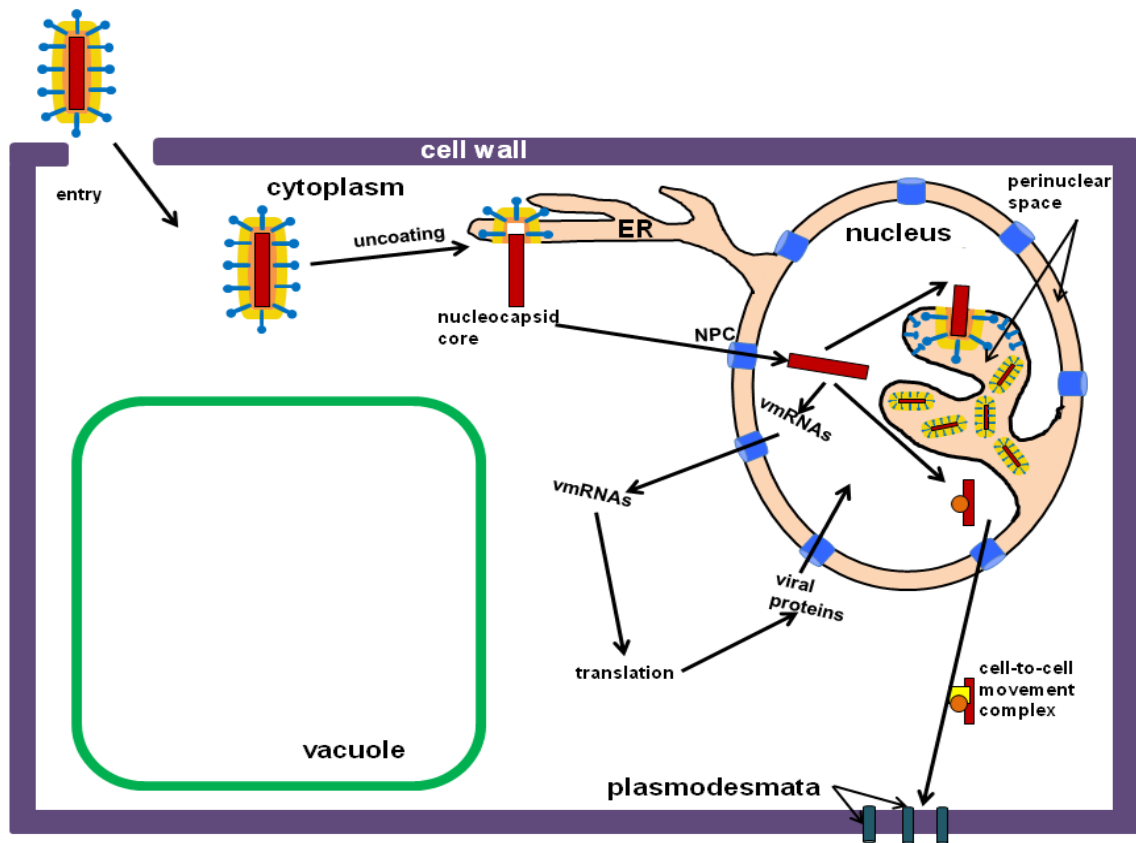


FIGURE 1.1 Replication cycle of PYDV

The main pathway for viral entry of PYDV is due to insect feeding, at which point the virus can enter the cell cytoplasm or vacuole. Uncoating is believed to take place on the ER membranes. After the nucleocapsid core is released into the cytoplasm, it is transported to the nucleus through nuclear pore complexes (NPC). Viral mRNAs are then transcribed, and exported to the cytoplasm for translation. The viral proteins are then imported back into the nucleus, where they participate in replication and the formation of viroplasms. At this stage, nucleocapsid cores are likely associated with PYDV-M and Y proteins, and are transported to the plasmodesmata where cell-to-cell movement occurs. Morphogenesis occurs when M protein condenses the viral nucleocapsids and associates with viral G protein embedded in the inner nuclear membrane. The mature viral particle then buds through the inner nuclear membrane and accumulates in the perinuclear space.

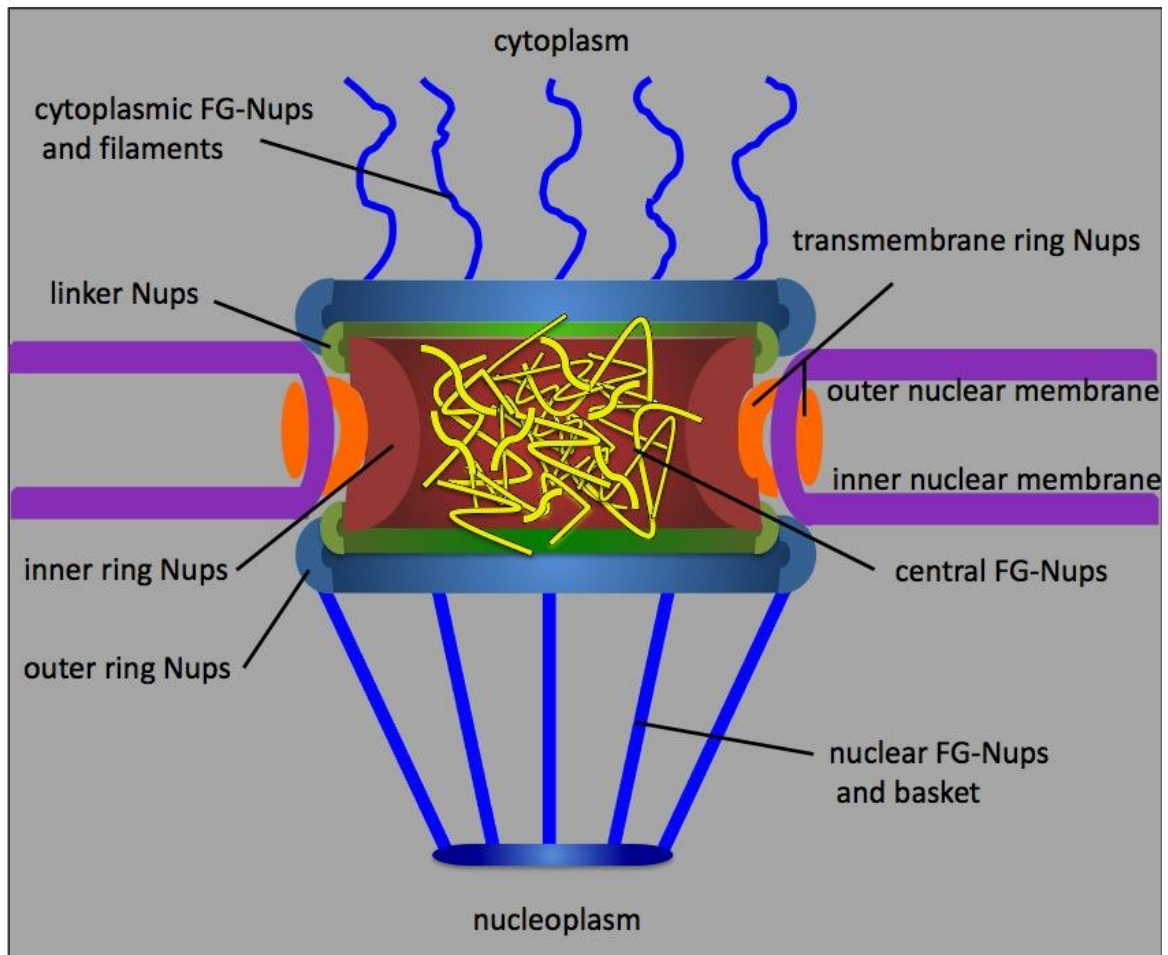


FIGURE 1.2. Schematic of a nuclear pore complex.

The NPC is divided into seven functional groups. The symmetrical core consists of the inner ring Nups, the outer ring Nups, the transmembrane ring Nups, the linker Nups, and the central FG-Nups. The cytoplasmic face of the NPC consists of the cytoplasmic FG-Nups and filaments. The nuclear side of the NPC is composed of the nuclear FG-Nups and the basket.

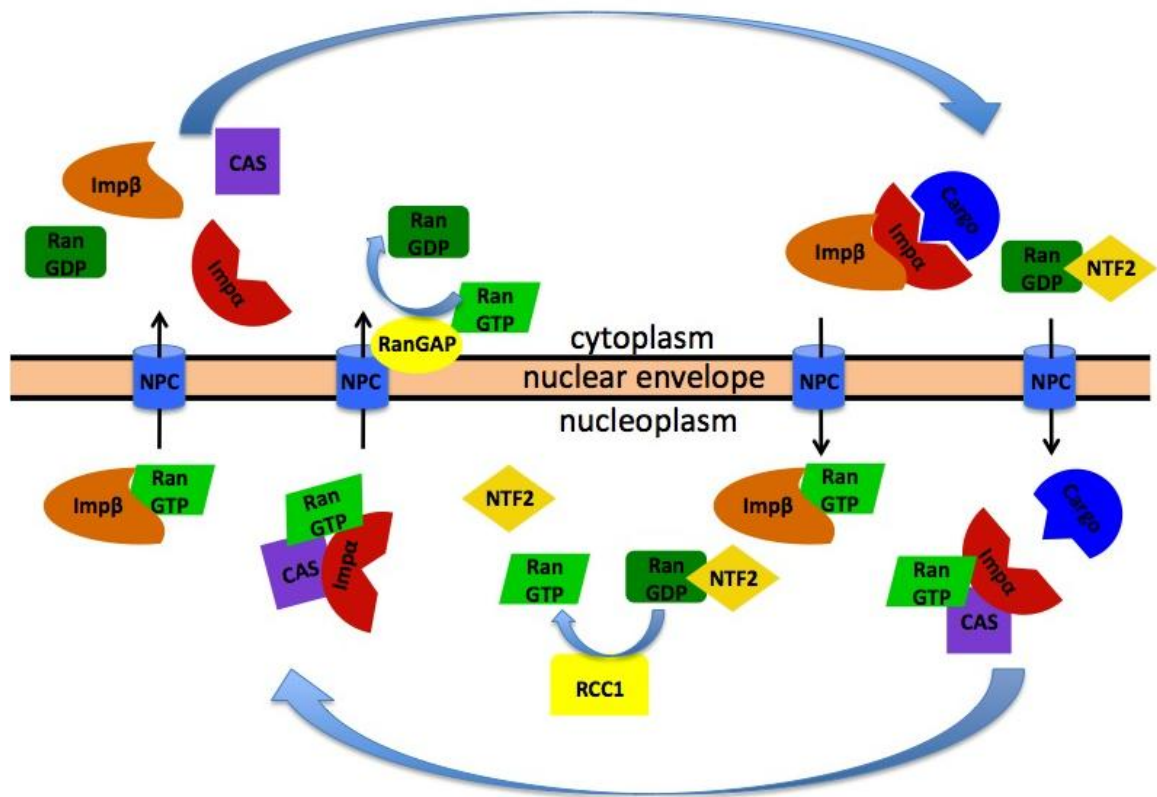


FIGURE 1.3. The classical nuclear import pathway and recycling of import factors.

In the cytoplasm, importin- α and importin- β form a trimeric complex with an NLS-containing cargo protein (upper right). The complex passes through the nuclear pore complex (NPC) into the nucleus. RanGTP binds to importin- β and releases the complex. RanGTP and the export factor CAS facilitate release of the cargo protein from importin- α . The RanGTP/importin- β and RanGTP/CAS/importin- α complexes are exported back through the NPC. RanGAP in the cytoplasm hydrolyzes RanGTP to RanGDP, which leads to the dissociation of the export complex. RanGDP is imported to the nucleus via the nuclear transport factor NTF2. The nucleotide exchange factor RCC1 converts RanGDP to RanGTP, which leads to dissociation of NTF2.

CHAPTER II¹

The nucleocapsid protein of *Potato yellow dwarf virus*: protein interactions and nuclear import mediated by a non-canonical nuclear localization signal

Rhabdoviruses are structurally complex enveloped viruses, the plant-adapted members of which are separated into the genera *Cytorhabdovirus*, which replicate in the cytoplasm of infected cells, and *Nucleorhabdovirus*, the viruses considered here, which replicate in nuclei of infected cells. The minimal infectious unit of these viruses is a nucleocapsid composed of viral genomic single-stranded negative-sense RNA encapsidated over its entire length by a nucleocapsid (N) protein and sub-molar amounts of an RNA-dependent RNA polymerase composed of a large (L) protein and a phosphoprotein (P) (Goodin et al., 2001; Jackson et al., 2005; Ammar el et al., 2009). The N proteins of viruses belonging to the genus *Nucleorhabdovirus* contain nuclear localization signals (NLSs) that not only direct the protein itself to the nucleus but also the viral nucleocapsid, which is required to establish sites of replication and viral assembly in nuclei of infected plant cells (Wagner et al., 1996; Wagner and Jackson, 1997; Jackson et al., 2005; Kuzmin et al., 2009). In this regard, the N protein-mediated transport of nucleocapsids parallels the role of VirE2 in the transfer of transfer DNA (T-DNA) from *Agrobacterium tumefaciens* to nuclei of plant cells to which it is attached. In broader terms, the nuclear transport and subsequent cell-to-cell movement of rhabdoviral nucleocapsids provides insight into the general macromolecular trafficking in plant cells. As such the identification and mapping of NLSs in N proteins is critically required to understand the mechanism by which these proteins, and their ribonucleoprotein

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complexes are targeted to the nuclei (Citovsky et al., 1992;Citovsky et al., 2004;Deng et al., 2007).

In this study, we report the characterization of the NLS responsible for nuclear targeting of the nucleocapsid protein of *Potato yellow dwarf virus* (PYDV), which is the type species of the nucleorhabdovirus genus (Hsu and Black, 1973; 1974;Bandyopadhyay et al., 2010). Nucleocapsids of these insect-vectored viruses are transported to the nucleus where they establish sites of replication known as viroplasms. These viroplasms are spatially separated from the sites of virion assembly, which takes place on the inner nuclear membrane, with condensed nucleocapsids accumulating in the perinuclear space of infected cells (Goodin et al., 2001;Goodin et al., 2007;Martin et al., 2009). In order to initiate new rounds of the infection cycle, nucleocapsids must exit the nucleus, via a mechanism that is thought to involve multiple host factors, microtubules and membranes (Min et al., 2010).

We have shown that despite the fact that protein localization prediction algorithms failed to identify an NLS in PYDV-N, this protein is fully capable of directing green fluorescent protein (GFP) exclusively to the nucleus in plant cells expressing GFP:PYDV-N fusions (Bandyopadhyay et al., 2010). Additionally, we have shown that PYDV-N interacts with one of two isoforms of the karyophilic transporter, importin- α , in plant cells (Martin et al., 2009). Finally, we have shown that PYDV-N interacts with the nuclear localized matrix (M) and P proteins of PYDV, thus raising the possibility that these two proteins may in fact play a role in facilitating the nuclear transport of N (Bandyopadhyay et al., 2010). While these studies contribute to an understanding of

plant-virus interactions, the identification of novel NLSs aids the categorization of the nuclear transport signals that are able to function in plants. As such, this provides a means to improve predictive algorithms for identifying plant proteins associated with nuclei (Kosugi et al., 2009).

METHODS

Plant materials and virus maintenance

All plants, including transgenic marker lines, were maintained in greenhouse on open benches under ambient conditions (Martin et al., 2009). PYDV (American Type Culture Collection accession PV-234) was maintained by serial passage in *Nicotiana benthamiana* and *N. rustica* in insect-proof cages under ambient conditions.

Generation of site-directed mutants

Site-directed mutagenesis was performed using the Change-IT™ Multiple Mutation Site Directed Mutagenesis Kit (USB) according to protocols provided by the manufacturer. All mutagenesis was conducted using sequence-validated portions of the PYDV-N open reading frame cloned into plasmid pDONR221 (Invitrogen). Mutagenized clones were validated by DNA sequencing to confirm the presence of specific mutations.

Protein expression in plant cells

Expression of autofluorescent protein fusions in plant cells for localization and bimolecular fluorescence complementation (BiFC) assays, were performed as

described previously (Chakrabarty et al., 2007; Goodin et al., 2007; Martin et al., 2009). In this study, the vector pSITE-2C (GFP-fusion) was used for localization assays, and pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays. Recombinant vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted as described previously (Goodin et al., 2002). Each expression construct was examined in 25 mm² leaf pieces taken from a minimum of two leaves, from a minimum of two independent plants. Multiple micrographs representative of several hundred examined cells were acquired for documentation.

Laser scanning confocal microscopy

All microscopy was performed on an Olympus FV1000 laser-scanning confocal microscope as described previously (Goodin et al., 2005). Micrographs for dual-color imaging were acquired sequentially, as described in Goodin et al., 2007.

Nuclear import assays in yeast

Sequence validated clones in pDONR221 (Invitrogen) were recombined into pNIA-DEST plasmids (Bandyopadhyay et al., 2010). Recombinant pNIA-DEST plasmids were transformed into *Saccharomyces cerevisiae* strain L40 (Zaltsman et al., 2007). Transformed yeast were grown at 30° C for 3 days, on minimal medium lacking tryptophan (Trp-). Colonies were then re-streaked onto minimal medium lacking both tryptophan and histidine (His-), containing 5mM 3-amino-1,2,4-triazole (3AT). Growth of yeast cultures on Trp-/His- medium indicates a functional nuclear localization signal in proteins expressed from pNIA-DEST (Zaltsman et al., 2007).

RESULTS

A nuclear localization signal (NLS) is present in the C-terminus of PYDV-N

We have reported previously that PYDV-N localizes exclusively to nuclei when expressed as a GFP-fusion in transgenic *N. benthamiana*, despite the absence of a predictable nuclear localization signal (Bandyopadhyay et al., 2010). To determine the karyophilic region of PYDV-N we expressed six overlapping fragments of PYDV-N as GFP fusions, and determined their subcellular localization *in planta*. Fragments 1-5 were found throughout the cell or excluded from the nucleus, whereas the C-terminal Fragment 6 (aa 350-473) was found to localize exclusively in the nucleus. In order to define the location of the NLS in PYDV-N we used alanine-scanning mutagenesis in the context of full-length N protein. A subset of these mutations, shown in Figure 2.1, demonstrated the location of the PYDV-N NLS between amino acids 419-434. Scanning mutagenesis upstream or downstream of this region did not influence nuclear localization. In particular, two QKRA repeats (⁴¹⁹QKRA⁴²² and ⁴³¹QKRA⁴³⁴) appeared to be required for NLS function (Figure 2.1 D-E and J-K), whereas the eight amino-acid residues separating them could be mutated without affecting nuclear localization (Figure 2.1 F-I). Fine mapping using single amino acid mutations within the QKRA motifs (Figure 2.2), revealed that amino acids R⁴²¹, K⁴³² and R⁴³³ are all required for NLS function (Figure 2.2 E, I and J).

Interaction of PYDV-N mutants with Importin- α

The initial mutagenesis and protein localization revealed two QKRA repeats that appeared to be required for nuclear localization of PYDV-N. This arrangement of amino acids, though not predicted by protein localization algorithms, were reminiscent of bipartite NLSs that interact with the nuclear import receptor importin- α , two isoforms of which are known to exist in *N. benthamiana* (Kanneganti et al., 2007). We have shown previously that PYDV-N interacts preferentially with *N. benthamiana* importin- α 1 (Martin et al., 2009). Therefore, to investigate the role of the QKRA motifs on NLS function, we conducted BiFC assays to test the interaction of PYDV-N mutants and *Nbimportin- α 1*. (Figure 2.3). GST was used as a negative-binding control as it has no interaction with *Nbimportin- α 1* (Figure 2.3, A1-3). Conversely, wild-type PYDV-N interacted strongly with *Nbimportin- α 1* (Figure 2.3B). However, PYDV-N mutants that failed to localize to the nucleus (see Figures 2.1 and 2.2) also failed to interact with *Nbimportin- α 1* (Figure 2.3, panels D, E, J and K). In addition, the mutants that were still localized to the nucleus when expressed *in planta* as GFP-fusions interacted with *Nbimportin- α 1* in the nucleus (Figure 2.3, panels C, F, G, H, I, and L).

Nuclear import of PYDV-N mutants is confirmed by a yeast-based assay

To confirm the *in planta* localization data for PYDV-N and mutants thereof, a yeast-based nuclear import assay (NIA) was employed (Figure 2.4). In this assay, only proteins containing a functional NLS will facilitate the nuclear import of a transcriptional activator required for expression of a reporter gene in yeast cells

(Zaltsman et al., 2007). Consistent with the *in planta* localization patterns, PYDV-N, and PYDV-N mutants Q⁴¹⁹A, K⁴²⁰A, A⁴²²G, Q⁴³¹A, and A⁴³⁴G, were positive in the NIA. PYDV-N mutants R⁴²¹A, K⁴³²A, and R⁴³³A were NIA-negative. In addition, the potential PYDV-N NLS, aa 419-434, was NIA-positive, whereas the same sequence containing the QK⁴³¹⁻⁴³²AA double mutant was NIA-negative.

The region spanning aa 419-434 of PYDV-N is sufficient to direct nuclear localization

In order to test function of the PYDV-N NLS outside the context of the full-length protein, aa 419-434 of PYDV-N were expressed as a fusion to GFP in plant cells. The addition of the PYDV-N aa 419-434 was able to localize GFP exclusively to the nucleus (Figure 2.5, A-I). In addition, when BiFC was performed between PYDV-N aa 419-434 and *Nbimportin-α* 1, they were shown to interact and the interaction was localized to the nucleus (Figure 2.5, J-R).

The PYDV N-N self-interaction may occur after nuclear import

It has previously been shown that PYDV-N interacts with itself, PYDV-P, and PYDV-M in the nucleus (Bandyopadhyay et al., 2010). Such interactions may occur prior to or following nuclear import. To investigate if a PYDV-N containing a functional NLS could interact with and facilitate the nuclear import of NLS mutants, BiFC was performed between PYDV-N and N-mutants (Figure 2.6). It was found that the mutants that retained the ability to be imported into nuclei (Figure 2.6 C, F, G, H, I and L) were also capable of interaction with PYDV-N in the nucleus. In contrast,

mutants containing a non-functional NLS did not interact with PYDV-N (Figure 2.6 D, E, J, and K).

To determine if the absence of interaction between PYDV-N and mutants QK⁴¹⁹⁻⁴²⁰AA, RA⁴²¹⁻⁴²²AA, QK⁴³¹⁻⁴³²AA, and RA⁴³³⁻⁴³⁴AA, was due to a disruption of the N-interacting domain, BiFC was performed to test self-interaction of these mutants. This showed that although these mutants no longer interact with wild-type PYDV-N, they are able to self-interact and interact with each other, and this interaction occurs outside of the nucleus (Figure 2.7J-L). In addition, BiFC between PYDV-N and the overlapping fragments of PYDV-N showed that only the N-terminal fragment, Fragment 1, showed interaction with PYDV-N (Figure 2.8C), suggesting that the N-N interaction domain is contained in this fragment. Taken together, these results suggest that the PYDV-N self-interaction occurs after nuclear import.

N-NLS mutants cannot be ferried into the nucleus by the PYDV phosphoprotein or matrix protein.

BiFC assays using wild-type and NLS mutants of PYDV-N suggested that this protein does not enter the nucleus as homo-oligomeric complexes. To address the possibility that other interaction partners may play a role in the nuclear import of N, we conducted BiFC assays with the N mutants and PYDV-P or -M.

Assays with PYDV-P showed, contrary to expectation, that NLS mutants of N were capable of interacting with PYDV-P and in doing so retarded the nuclear import of PYDV-P (Figure 2.9). Mutants that were no longer localized to the nucleus

as GFP-fusions expressed in plant cells (Figure 2.9 D, E, J, K) showed interaction with PYDV-P completely outside of the nucleus.

In a similar fashion, N-NLS mutants retained their ability to interact with PYDV-M (Figure 2.10). N proteins that were competent for nuclear import interacted with N on intranuclear sites (Figure 2.10 C, F, G, H, I and L). In contrast, N proteins with a non-functional NLS interacted with M at the periphery of nuclei and not in intranuclear loci (Figure 2.10 D, E, J, K).

DISCUSSION

At present, determination of the subcellular localization of proteins for organisms whose genomes have been sequenced, is based most frequently on the use of predictive algorithms and, to a far lesser extent, actual protein localization data. The consequence of this is likely to be extreme underestimation in the number of proteins that are targeted to the nucleus as algorithms used to predict nuclear localization are designed primarily to look for basic, arginine/lysine-rich regions, typical of proteins imported into the nucleus by importin α/β -mediated pathways (Cokol et al., 2000). However, many novel non-basic nuclear localization signals (NLSs) have been identified, including the M9 and other non-canonical NLSs that are not detected using the most popular predictive algorithms (Mattaj and Englmeier, 1998; Kumar and Raghava, 2009). The situation becomes more complex if we consider the fact that no less than ten different subnuclear loci, which have markedly different functions, have been identified (Spector, 2006). Localization to these loci is not predicted by publicly available algorithms, which are limited to predicting only nuclear or nucleolar localization (Nakai and Horton, 2007; Kumar

and Raghava, 2009).

In the present study we have used protein interaction and *in vivo* localization to map and characterize the bipartite NLS of PYDV-N. Although this NLS could not be predicted by *in silico* methods, our *in vivo* analyses provide comprehensive evidence that the PYDV-N-NLS is located between the amino acids 419-433 of the mature N protein. Mutagenesis scanning employing double mutants revealed that repeated QKRA motifs were essential for nuclear localization. However, fine mapping where single amino acids were mutated showed that the most critical residues were R⁴²¹, K⁴³² and R⁴³³ located within the two QKR repeats. This apparent discrepancy might be due to greater perturbation of NLS function in the double mutants compared to the single mutations, which revealed only those residues that had the greatest impact on nuclear import and on binding to importin- α . Ultimately, the PYDV-N-NLS was determined to be ⁴¹⁹QKRANEEAPPAAQKR⁴³³, which is generally consistent with the consensus KRX₁₀₋₁₂K(K/R)(K/R) found in bipartite NLSs that function in plants (Kosugi et al., 2009). A key difference however is the lack of a third basic residue after the spacer region, suggesting that a basic amino acid at this site is not critical to the function of some NLSs. Importantly, although plants and yeast encode different numbers of importin- α isoforms, both *in planta* and yeast-based nuclear import assays returned similar data thus further validating the utility of yeast to screen plant nuclear localized proteins (Zaltsman et al., 2007).

Comparison to other predicted or physically-mapped NLSs in nucleorhabdoviral N proteins, for which sequence data are available, it is apparent that N-NLSs are

found in all cases, thus far, near the carboxy termini of these proteins (Table 2.1A). The reason for this may be that, where mapped, the N-N interaction domain resides in the amino-termini of these proteins (Goodin et al., 2001). Additionally, in the majority of cases, the N-NLSs are of the bipartite class. Importantly, the PYDV-N NLS is not restricted to this virus but is also found in a plethora of Arabidopsis proteins with known or predicted functions in nuclei, suggesting that the QKR motif is an important class in plant NLS (Table 2.1B).

Our BiFC data suggest that N-NLS mutants retain the ability to self-associate and that wild-type N proteins could not facilitate nuclear import of mutant proteins. It is likely therefore that wild-type N proteins do not self-associate until they have entered the nucleus. In contrast, the N-NLS mutants were able to inhibit the nuclear import of P proteins, suggesting that the N:P interaction domain overlaps with the P-NLS. This finding could have consequences for engineering resistance to protect plants from rhabdovirus infections. Not only may N proteins incapable of nuclear import serve as dominant-negative mutants of N function, but by interfering with P may provide an additional level of inhibition of critical viral functions. Curiously, the interaction of N-NLS mutants with the M protein occurred at punctate loci on the periphery of nuclei suggestive of the location of nuclear pore complexes (NPCs;(Fiserova et al., 2009)). In the absence of other viral proteins, the PYDV-M protein induces the intranuclear accumulation of the inner nuclear membrane (Martin et al., 2009;Bandyopadhyay et al., 2010). Why an N-NLS mutant would inhibit this process will likely require identification of additional components of the NPC in *N. benthamiana*, as has recently been done for Arabidopsis (Meier and

Brkljacic, 2009; Tamura et al., 2010; Tamura and Hara-Nishimura, 2011).

TABLE 2.1. Predicted NLSs in Rhabdovirus nucleocapsid proteins.

A. Nuclear localization signals in nucleocapsid proteins of nucleorhabdoviruses.

Only the SYN and PYDV signals have been mapped physically, others have been predicted using in silico methods. The numbers indicate the amino acid residues in the full-length proteins that mark the NLSs. **B.** The PYDV-N-NLS was used as a probe in BLASTp searches to identify Arabidopsis proteins with QKR-motif NLSs. The top 10 hits are shown. MFSV – Maize fine streak virus; RYSV – Rice yellow stunt virus; MMV – Maize mosaic virus; IMMV – Maize Iranian mosaic virus; TaVCHV – Taro vein chlorosis virus.

A

Virus	Type	NLS
MFSV	Bipartite	⁴³⁶ KRSSDGTGNVSKKKSRK ⁴⁵²
SYNV	Bipartite	⁴⁶⁵ PSRKRRSDALTTEKPKK ⁴⁸¹
RYSV	Bipartite	⁴⁰⁵ KKLGPPRANAHSRRKEP ⁴²¹
MMV	Monopartite	⁴³⁸ PAPKKTR ⁴⁴⁴
IMMV	Monopartite	⁴³² PRLRRGS ⁴³⁸
TaroVCHV	Monopartite	⁴²² PTKKRTW ⁴²⁸
PYDV	Bipartite	⁴¹⁹ QKRANEEAPPAAQKR ⁴³³

B

Accession Number	Description	Sequence
EU183122	PYDV-N	QKRANEEAPPAAQKR
dbj BAD95377.1	RING finger-like protein	QKRKRCVEGESSGKR
dbj BAF01759.1	Putative non-LTR reverse transcriptase	QKRINGWTSKFLSKR
gb AEC08614.1	RAD50 DNA repair protein	QKRAKDEIKMGISKR
gb AEE85106.1	Ftsj-like methyltransferase family protein	QKRVDRRMKSDDRKR
dbj BAA96899.1	DNA polymerase III catalytic subunit	QKRVSRYAAWLSLKR
gb AEE30605.1	Transcription factor MEE8	QKRSAESRREGKKKR
gb AEE79234.1	F-Box protein	QKRKRCVEGESSGKR
gb AED90531.1	E3 ubiquitin-protein ligase UPL4	QKRMEVVEELPADKR
gb AEE30120.1	zinc finger CCCH domain-containing protein	QKRNPDLSDSSNLKR
ref NP_188215.1	UDP-glycosyltransferase-like protein	QKRSGTCDRKSDFKR

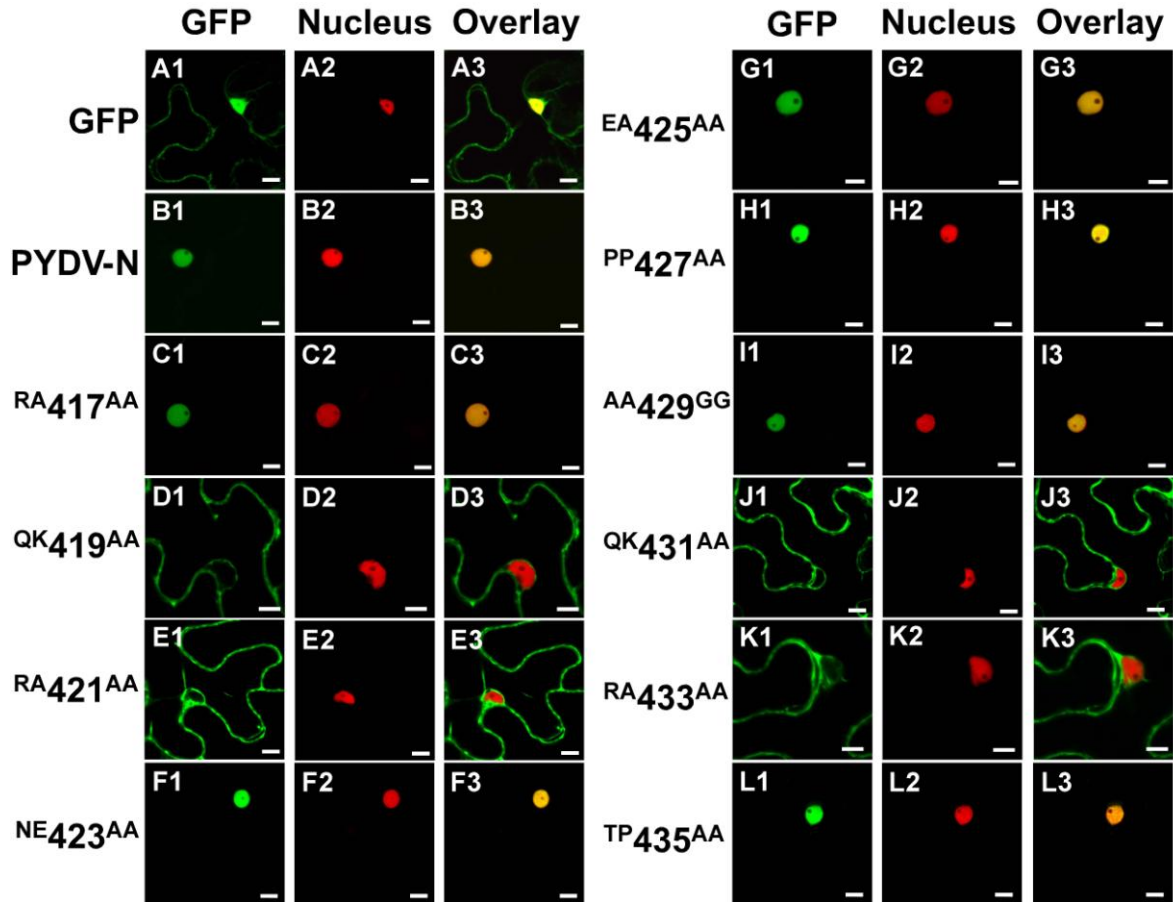


FIGURE 2.1. Localization of double mutants of PYDV-N.

Single-plane confocal micrographs of GFP:protein fusions expressed transiently in leaf epidermal cells of *N. benthamiana* plants transgenic for the nuclear marker, RFP:histone 2B. Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP nuclear marker, and the resultant overlay, respectively. (A1-3) GFP only. (B1-3), PYDV-N. (C-L) are PYDV-N site-directed double mutants. Numbers indicate the amino acid residue in the full-length N protein where a two-residue change to alanine was introduced. Scale bar = 10 μ m.

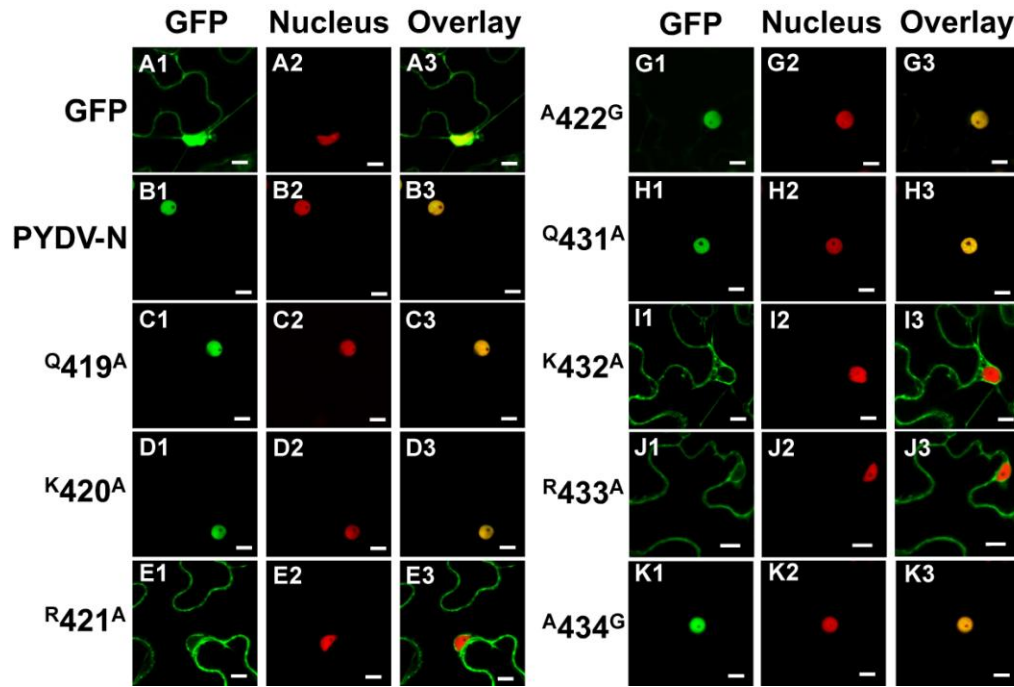


FIGURE 2.2 Localization of single amino acid mutants of PYDV-N.

Single-plane confocal micrographs of protein-fusions expressed in leaf epidermal cells of transgenic *N. benthamiana* plants expressing RFP fused to a nuclear marker, histone 2B. Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP nuclear marker, and the resultant overlay, respectively. **(A1-3)** GFP only. **(B1-3)** GFP:PYDV-N fusion. **(C-K)**, PYDV-N site-directed single mutants. Numbers indicate the amino acid residue in the full-length N protein where a single residue change to alanine was introduced. In cases where the residue was alanine in the native protein, changes to glycine were made. Scale bar = 10 μ m.

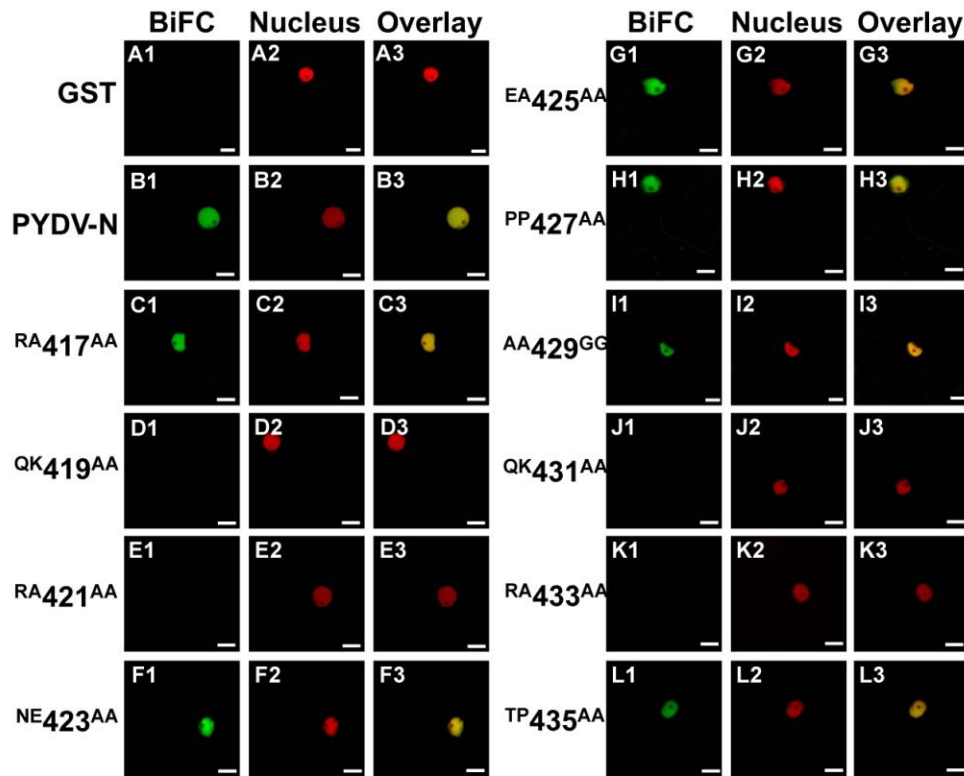


FIGURE 2.3. BiFC assay between PYDV-N and importin- α .

Confocal micrographs showing results of BiFC assays to determine interactions between Importin- α and PYDV-N mutants. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a CFP:nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), nuclear marker, and the resultant overlay, respectively. (A1-3) non-binding control GST. (B1-3) BiFC between Importin- α and PYDV-N. (C-L), BiFC between Importin- α and double mutants of PYDV-N, described in Figure 2.1. Scale bar = 10 μ m.

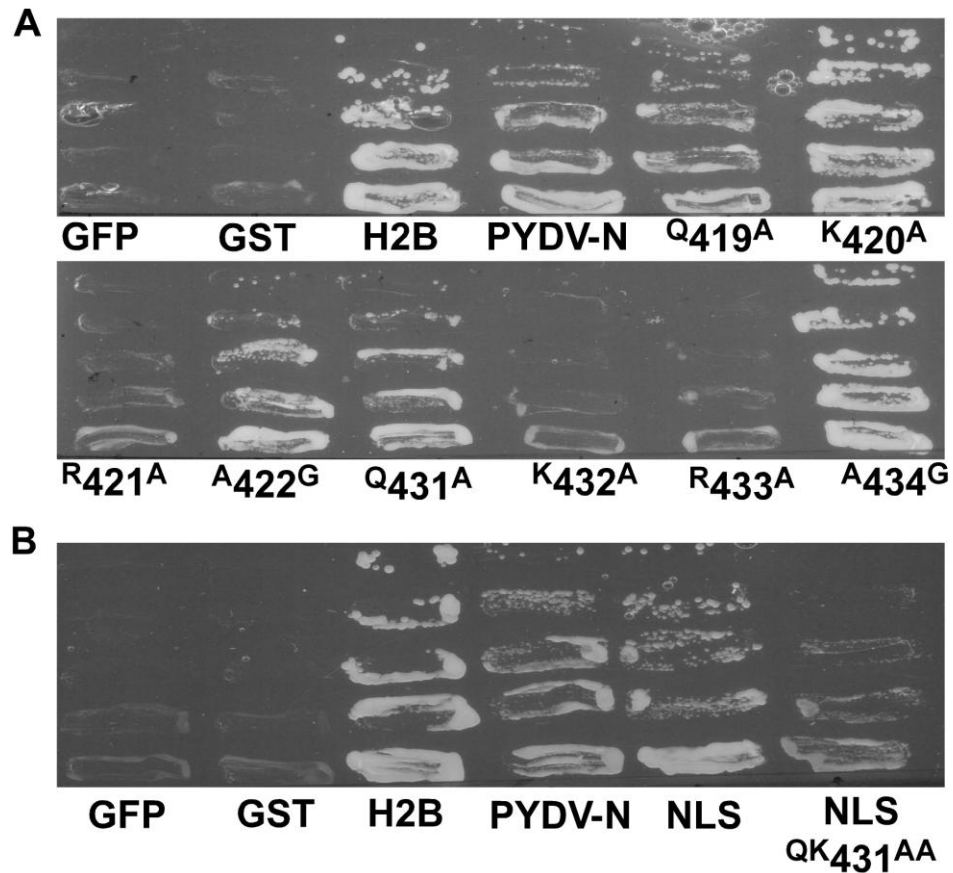


FIGURE 2.4. Yeast-based assay for identification of functional NLS in PYDV-N mutants.

Positive (H2B) and negative (GFP/GST) control proteins along with PYDV proteins (N, and site directed N-mutants) were expressed from pNIA-DEST in yeast strain L40, and grown on minimal media lacking histidine. Yeast growth on media lacking histidine is scored as a positive result in this assay, and indicates the presence of a functioning NLS.

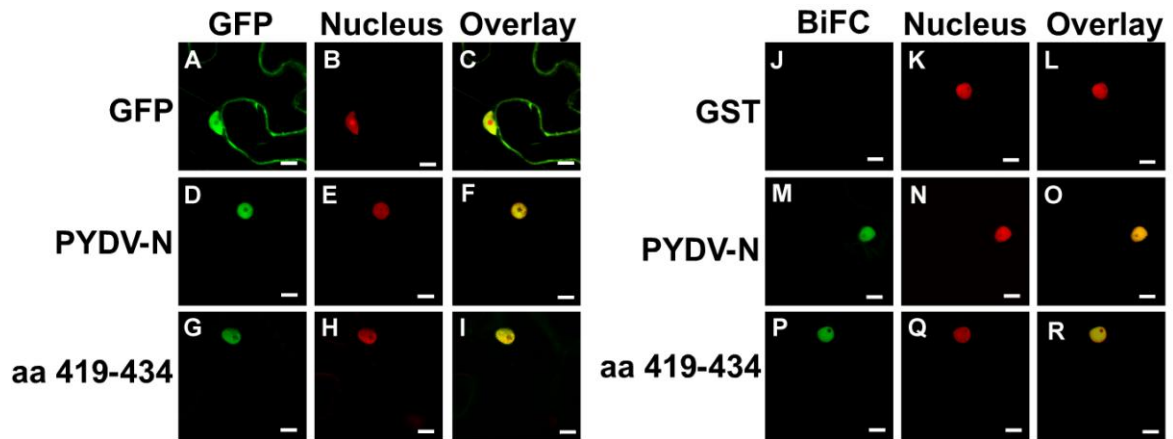


FIGURE 2.5. A 16 amino-acid sequence of PYDV-N can direct GFP to the nucleus.

Confocal micrographs of protein fusions expressed in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a fluorescent nuclear marker (Nucleus). Panels A-I show localization of GFP fusions while panels J-R show results of BiFC assays to test interactions with importin- α . (B, E, H, K, N, Q) fluorescent nuclear marker. (C, F, I) overlay of GFP-fusion and nuclear marker. (A) localization of GFP. (D) localization of full-length PYDV-N fused to GFP. (G) localization of the karyophilic domain of PYDV-N (aa 419-434) fused to GFP. (L, O, R) overlay of BiFC and nuclear marker. (J). BiFC interaction of GST and importin- α . (M) BiFC interaction of PYDV-N and importin- α . (P) BiFC interaction of aa 419-434 PYDV-N and importin- α . Scale bar = 10 μ m.

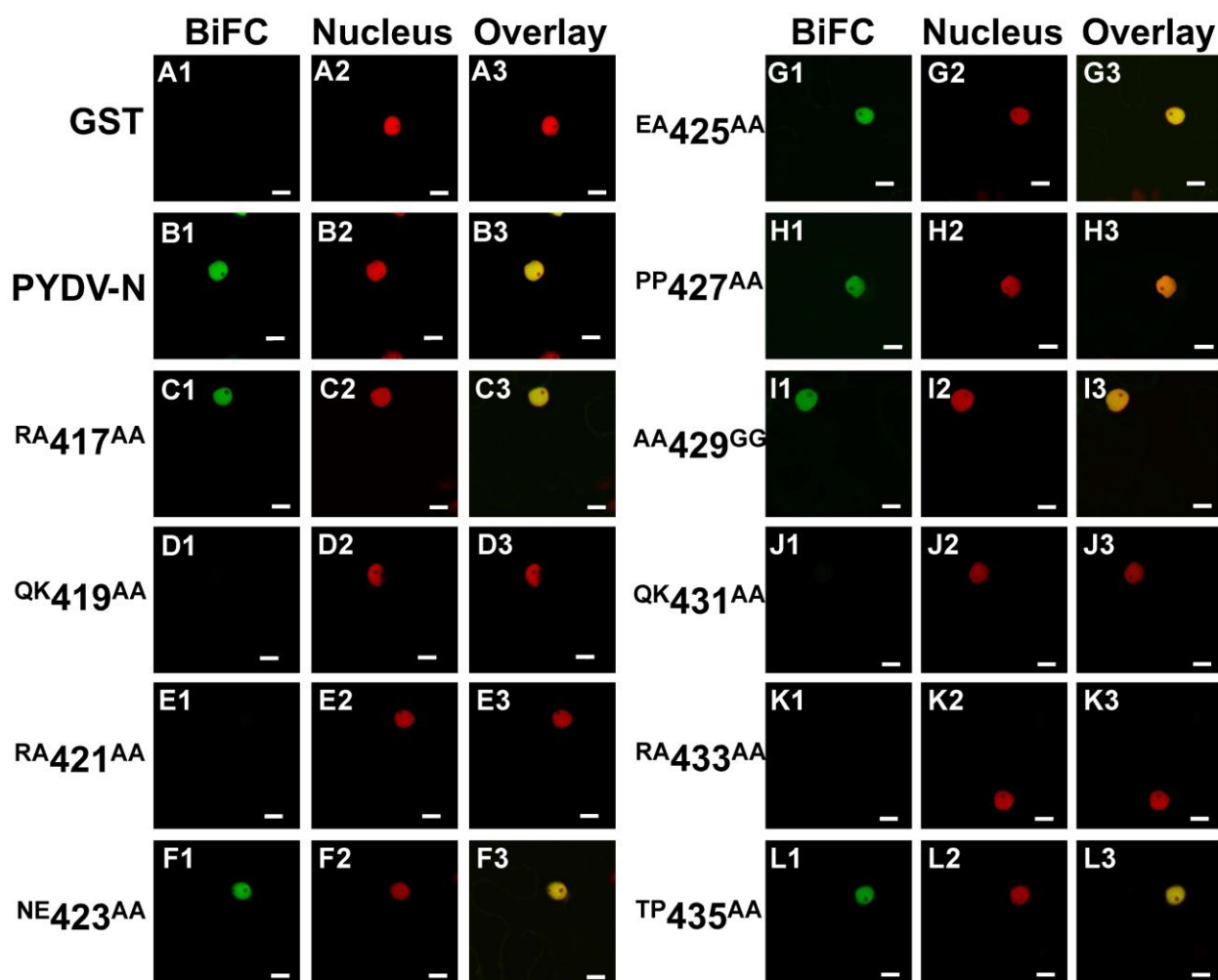


FIGURE 2.6. BiFC assay between PYDV-N and PYDV-N mutants

Confocal micrographs of BiFC assays testing the ability of PYDV-N mutant proteins to associate with the wild-type N protein. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker (Nucleus). Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. Shown here are BiFC assays conducted with wild-type PYDV-N expressed as a fusion to the carboxy-terminal portion of YFP and –N mutants expressed as fusions to the amino-terminal fragment of YFP. (A) GST, used as a non-binding control. (B) wild-type PYDV-N. (C-L) BiFC between PYDV-N and site-directed double mutants of PYDV-N shown in Figure 2.1. Scale bar = 10 μ m.

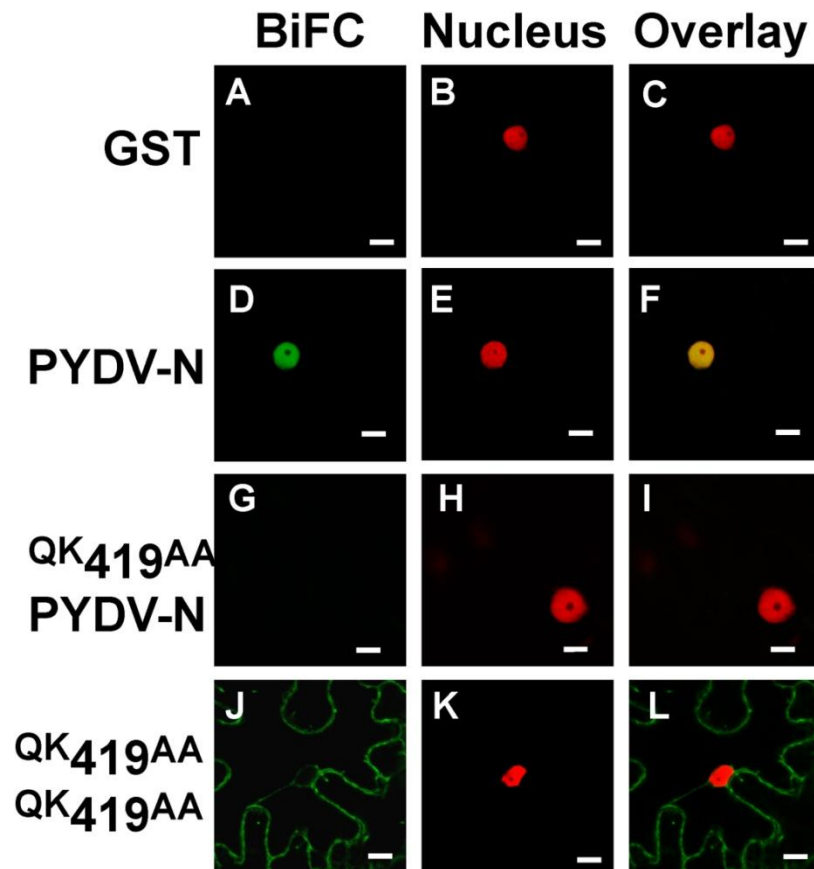


FIGURE 2.7. PYDV-N NLS mutants interact outside the nucleus.

Confocal micrographs showing results of BiFC assays to determine the ability of self-associate. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker (Nucleus; **B, E, H, K**). (**C, F, I, L**) Overlay of BiFC and nuclear marker. (**A**) BiFC between GST and PYDV-N. (**D**). BiFC self-interaction of wild-type PYDV-N. (**G**) BiFC between PYDV-N and an N mutant that is incapable of nuclear import. (**J**) BiFC self-interaction of ^{QK419AA} mutant of PYDV-N. Scale bar = 10 μ m.

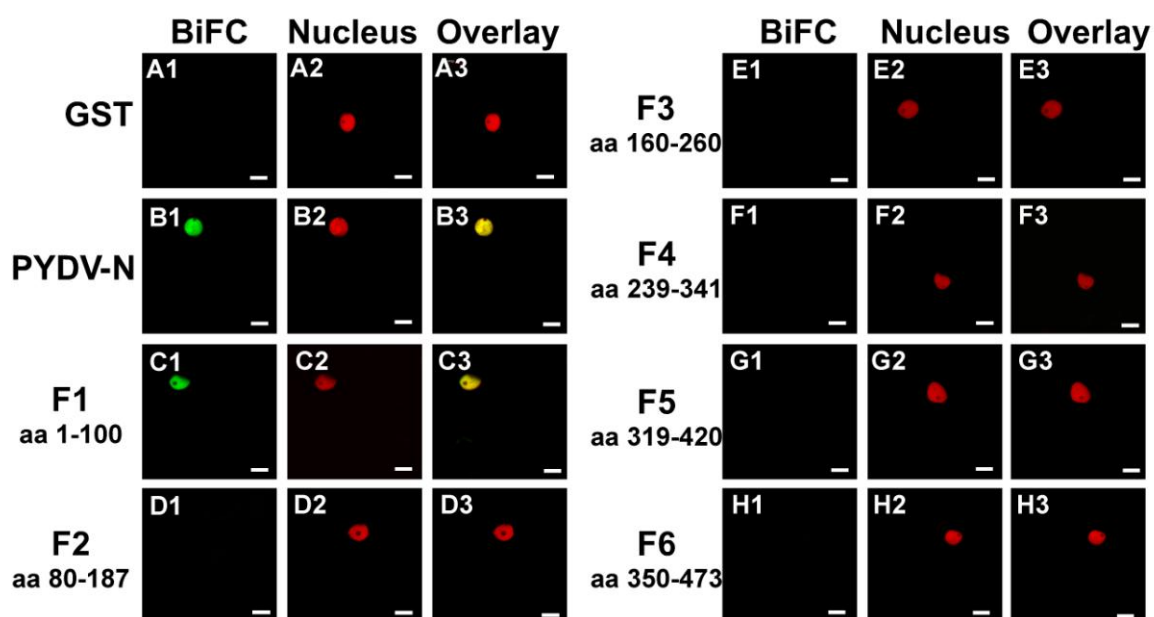


FIGURE 2.8. The PYDV-N interacting domain is in the N-terminal 100 amino acids.

Confocal micrographs showing PYDV-N fragment interactions with PYDV-N determined by BiFC. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker (Nucleus). Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. Shown is the BiFC assay of fragments of PYDV-N with wild-type N protein. (A1-3) BiFC of GST and PYDV-N. (B1-3) BiFC self-interaction of wild-type PYDV-N. (C-H), BiFC between PYDV-N and PYDV-N fragments F1-F6. The portions of PYDV N used in the BiFC assays are indicated by the amino-acid (aa) designation for each panel. Scale bar = 10 μ m.

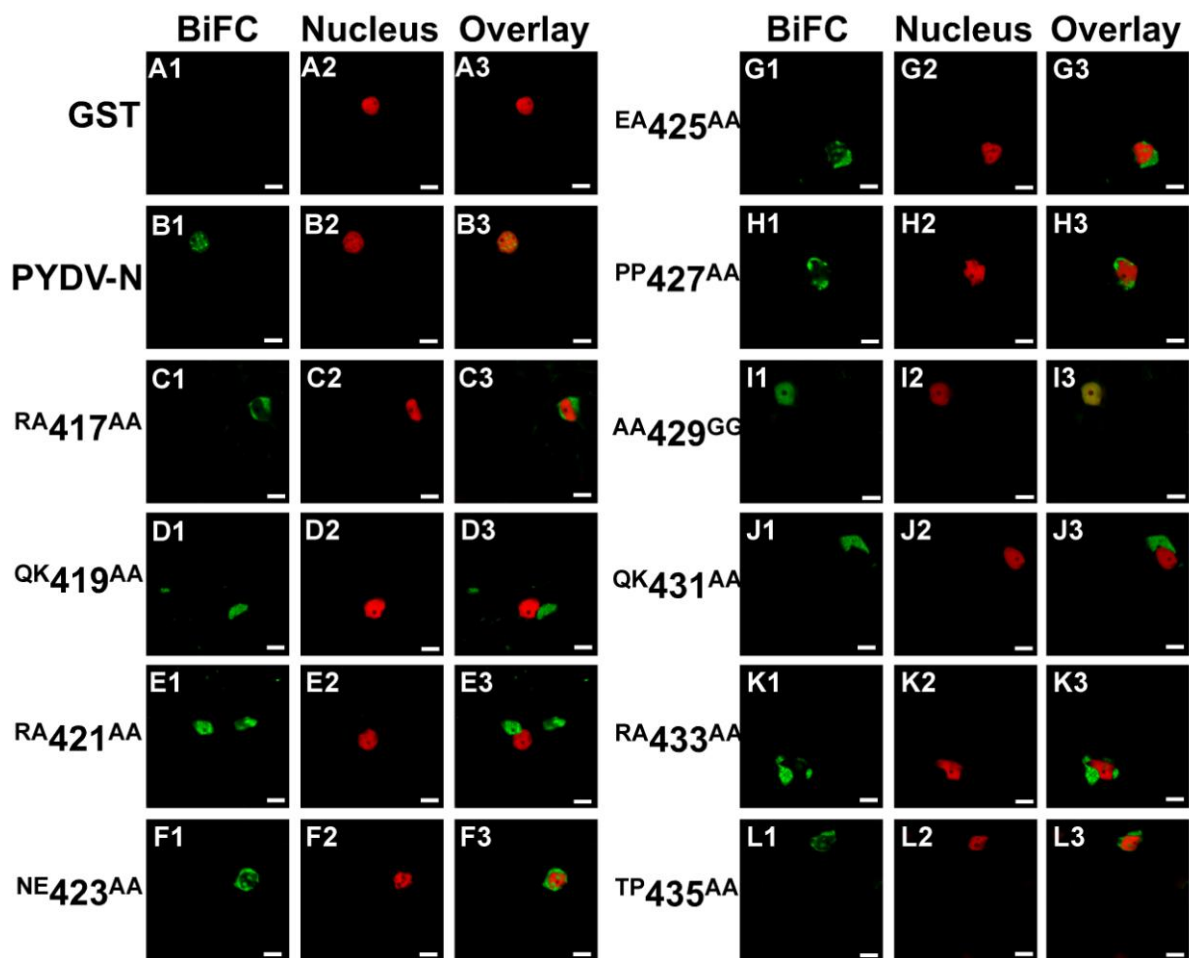


FIGURE 2.9. BiFC assay between PYDV-P and PYDV-N mutant proteins.

Confocal micrographs showing PYDV-N mutant protein interactions with PYDV-P determined by BiFC. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-P expressed as a fusion to the carboxy-terminal of YFP. (A1-3) BiFC between GST and PYDV-P. (B1-3) BiFC between PYDV-P and PYDV-N. (C-L) BiFC between PYDV-P and double mutants of PYDV-N. Scale bar = 10 μ m.

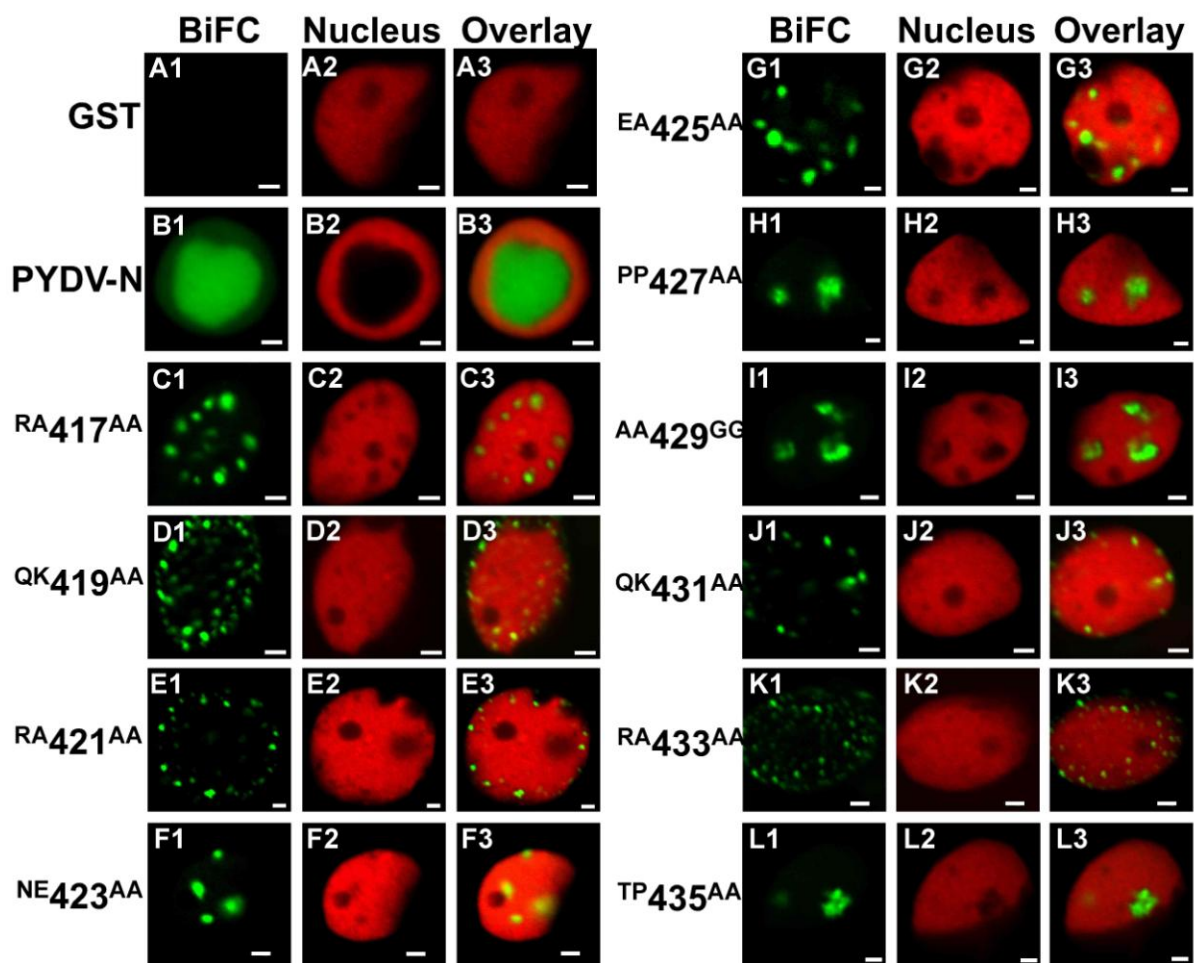


FIGURE 2.10. BiFC assay between PYDV-M and PYDV-N mutant proteins.

Confocal micrographs showing PYDV-N mutant protein interactions with PYDV-M determined by BiFC. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker (Nucleus). Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-M expressed as a fusion to the carboxy-terminal of YFP. (A1-3) BiFC of GST and PYDV-M. (B1-3) BiFC between PYDV-M and PYDV-N. (C-L) BiFC between PYDV-M and double mutants of PYDV-N. Scale bar = 2 μ m.

CHAPTER III

Mapping the domain responsible for nuclear import of the *Potato yellow dwarf virus* phosphoprotein

The Rhabdoviridae are a family of minus-sense, single-stranded RNA viruses containing members able to infect humans, mammals, fish, insects, and plants (Kuzmin *et al.*, 2009). The plant-adapted members are assigned to two genera based on their sites of replication and morphogenesis. Members of the genus *Cytorhabdovirus* replicate and undergo morphogenesis in the cytoplasm of infected cells. *Potato yellow dwarf virus* (PYDV) is type species of the second genus, *Nucleorhabdovirus*, members of which replicate in nuclei of infected cells (Jackson *et al.*, 2005). The minimal infectious unit of these viruses is a nucleocapsid composed of genomic negative-sense RNA encapsidated over its entire length by a nucleocapsid (N) protein, along with a polymerase (L) and a phosphoprotein (P; Goodin *et al.*, 2001; Jackson *et al.*, 2005; Ammar *et al.*, 2009). During morphogenesis, the nucleocapsid is condensed into viral cores by the matrix (M) protein, which then bud through the inner nuclear membrane into the perinuclear space (Goodin *et al.*, 2001, 2007; Martin *et al.*, 2009).

As replication and morphogenesis of PYDV occurs inside the nucleus, PYDV proteins synthesized in the cytoplasm must contain functional nuclear localization signals (NLSs) that directs these protein to the nucleus. While NLSs have been identified or predicted in the N proteins of several Nucleorhabdoviruses (Anderson *et al.*, 2012; Goodin *et al.*, 2001), less data are available for the P proteins. We have shown previously that PYDV-P is capable of directing green fluorescent protein

(GFP) exclusively to the nucleus when expressed in plant cells as a GFP:PYDV-P fusion (Ghosh *et al.*, 2003). In addition, *in silico* prediction (pSORTII; Horton and Nakai, 1997) has identified a possible monopartite NLS in PYDV-P, namely the amino acid sequence ⁸PSRKLRD¹⁴. However, it is important to confirm the predicted NLS function, as we have identified proteins with predicted NLSs that are not localized to the nucleus under steady state observation (Min *et al.*, 2010). In addition, many NLSs have been identified and characterized which are not detected using predictive algorithms (Mattaj and Englmeier, 1998; Kumar and Raghava, 2009; Anderson *et al.*, 2012).

In this study, we report the characterization of the NLS responsible for nuclear targeting of the phosphoprotein of *Potato yellow dwarf virus*. There are three possible scenarios by which a protein could be imported into the nucleus. A cargo protein containing a functional NLS can interact and be imported by a karyophilic transporter, such as importin- α . Secondly, a cargo protein may interact with another protein containing a functioning NLS, and in that way, can 'piggyback' into the nucleus. We have shown that PYDV-P interacts with the nuclear localized N protein, which raises the possibility that interaction with other PYDV proteins may play a role in facilitating nuclear transport of -P. Third, the nuclear import of PYDV-P could be mediated by one or more host factors, which control the timing and method of import. This study aims to improve predictive algorithms and understanding of plant-virus interactions by characterizing the NLS of PTDV-P and the impact on sub-cellular localization and interactions.

METHODS

Plant materials and virus maintenance

All plants, including transgenic marker lines, were maintained in greenhouse on open benches under ambient conditions (Martin *et al.*, 2009). PYDV (American Type Culture Collection accession PV-234) was maintained by serial passage in *Nicotiana benthamiana* and *Nicotiana rustica* in insect-proof cages under ambient conditions.

Generation of site-directed mutants

Site-directed mutagenesis was performed using the Change-IT™ Multiple Mutation Site-Directed Mutagenesis Kit (USB) according to protocols provided by the manufacturer. All mutagenesis was conducted using sequence-validated portions of the PYDV-P and PYDV-M open reading frames cloned into plasmid pDONR221 (Invitrogen). Mutagenized clones were validated by DNA sequencing to confirm the presence of specific mutations.

Protein expression in plant cells

Expression of autofluorescent protein fusions in plant cells for localization and bimolecular fluorescence complementation (BiFC) assays were performed as described previously (Chakrabarty *et al.*, 2007; Goodin *et al.*, 2007; Martin *et al.*, 2009; Anderson *et al.*, 2012). In this study, the vector pSITE-2C (GFP-fusion) was used for localization assays, and pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays, respectively. Recombinant vectors were transformed into *A. tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in

plant cells was conducted as described previously (Goodin *et al.*, 2002). Each expression construct was examined in 1 cm² leaf pieces taken from a minimum of two leaves, from a minimum of two independent plants. Multiple micrographs representative of several hundred examined cells were acquired for documentation.

Laser scanning confocal microscopy

All microscopy was performed on an Olympus FV1000 laser-scanning confocal microscope as described previously (Goodin *et al.*, 2005). Micrographs for dual-color images were acquired sequentially, as described in Goodin *et al.* (2007).

Nuclear import assay in yeast

Nuclear import assays were conducted as described previously (Bandyopadhyay *et al.*, 2010). Recombinant pNIA-DEST plasmids were transformed into *Saccharomyces cerevisiae* strain L40 (Zaltsman *et al.*, 2007). Transformed yeast were grown at 30°C for 3 days, on minimal media lacking tryptophan (Trp⁻). Colonies were then re-streaked onto minimal media lacking both tryptophan and histidine (His⁻), containing 50 mM 3-amino-1,2,4-triazole (3AT). Growth of yeast cultures on Trp⁻/His⁻ media indicates a functional NLS in proteins expressed from pNIA-DEST (Zaltsman *et al.*, 2007).

RESULTS

An NLS is present in the N-terminus of PYDV-P

We have previously shown that a GFP-fusion of PYDV-P localizes exclusively to the nucleus when expressed in transgenic *N. benthamiana* (Bandyopadhyay *et al.*,

2010). In addition, prediction algorithms have identified a possible monopartite NLS between amino acids 8-14 of PYDV-P (Horton and Nakai, 1997).

To determine the region of PYDV-P responsible for nuclear import of this protein, four overlapping fragments (named fA-fD; Figure 3.1) of PYDV-P were created, and expressed *in planta* as GFP fusions. As mentioned above, wild-type PYDV-P localizes exclusively to the nucleus (Figure 3.2A). Fragments fB-fD were found throughout the cell or excluded from the nucleus (Figure 3.2C-E), whereas the N-terminal fragment, fA, was localized exclusively to the nucleus (Figure 3.2B). In order to further define the residues responsible for nuclear localization, alanine-scanning mutagenesis was performed in the context of full-length protein. A change from nuclear to non-nuclear localization indicates the mutated amino acids are critical for nuclear import. Despite being a predicted monopartite NLS, there appeared to be three regions required for nuclear import: amino acids ⁶ISPSRKLRDR¹⁵, ¹⁸SK¹⁹, ³⁶KK³⁷ (Figure 3.3E-I, K, T, respectively). Substituting these amino acids for alanine resulted in a non-nuclear localization pattern, in contrast to the localization of wild-type PYDV-P (Figure 3.3B). However, fine mapping this region using single amino acid mutations did not identify any single amino acids required for NLS function. Scanning mutagenesis upstream or downstream of these mutants did not affect localization pattern.

The N-terminal 41 amino acids of PYDV-P are sufficient for nuclear import of PYDV-P

In order to test function of the identified PYDV-P NLS outside the context of the full-length protein, aa 1-41 of PYDV-P were expressed as a GFP-fusion in plant cells. Expression of GFP alone in plant cells results in localization throughout the cell, including (Figure 3.4A), and PYDV-P fused to GFP localizes exclusively to the nucleus (Figure 3.4B). Similarly, the addition of aa 1-41 of PYDV-P to GFP resulted in exclusively nuclear localization (Figure 3.4C).

PYDV-N is able to ferry PYDV-P mutants into the nucleus

Previous studies have shown that PYDV-N interacts with PYDV-P, and that this interaction occurs in the nucleus (Bandyopadhyay *et al.*, 2010). To investigate if PYDV-N can facilitate the nuclear import of PYDV-P mutants, BiFC was performed between PYDV-N and PYDV-P mutants (Figure 3.5). It was found that all mutants of PYDV-P retained the ability to interact with PYDV-N. Interestingly, all of the mutants except ^{AR22AA} (Figure 3.5M), including those that were non-nuclear as GFP-fusions, interacted with PYDV-N inside the nucleus (Figure 3.5C-L, N-V).

PYDV-P does not interact with *N. benthamiana* Importins, but does interact with two *A. thaliana* importin- α isoforms

In the classical nuclear import pathway, NLSs on cargo proteins interact with the nuclear import receptor importin- α , which has two known isoforms in *N. benthamiana* (Kanneganti *et al.*, 2007). We have previously shown that PYDV-N

preferentially interacts with *N. benthamiana* importin- α 1 (*Nbimportin- α 1*; Martin *et al.*, 2009), and have identified a non-canonical NLS in PYDV-N that interacts with *Nbimportin- α 1* outside the context of full-length protein (Anderson *et al.*, 2012). Therefore to investigate the role of the predicted NLS in PYDV-P, we conducted BiFC assays to test interaction of the predicted NLS outside the context of full-length protein with *Nbimportin- α 1*, 2, and *Nbimportin- β* . Results of this assay showed that wild-type PYDV-P did not interact with any of the *N. benthamiana* isoforms of importin- α or - β (Figure 3.6B). Similarly, the NLS of PYDV-P (aa 1-41), which localized completely in the nucleus as a GFP-fusion, did not interact with any of the *N. benthamiana* importins (Figure 3.6H). While *N. benthamiana* only codes for two importin- α isoforms, nine different isoforms of importin- α have been identified in *Arabidopsis thaliana*. Although *A. thaliana* is not a host for PYDV, we tested interaction between PYDV-P or the P-NLS and *Atimportin- α 1*, - α 4, - α 7, and - α 9. Results from this BiFC assay showed that neither PYDV-P nor the NLS interacted with *Atimportin- α 1* or - α 4 (Figure 3.6 C-D, I-J), but did interact with both *Atimportin- α 7* and - α 9 (Figure 3.6E-F, K-L). The PYDV-P mutants from the previous experiments were then tested for interaction with *Atimportin- α 7* and - α 9 (Figure 3.7). Only one PYDV-P mutant, ^{KK36AA}, no longer interacted with *Atimportin- α 7* (Figure 3.7F). Other mutants tested showed interaction and localization similar to that of wild-type PYDV-P (Figure 3.7C-E). Similar results were seen from the BiFC assay with *Atimportin- α 9*. Again, only the ^{KK36AA} mutant of PYDV-P no longer interacts with *Atimportin- α 9* (Figure 3.7L). PYDV-P double mutants with mutations

between aa 6-15 or 18-19 showed interaction with Atimportin- α 9, however, the localization of this interaction was completely outside the nucleus (Figure 3.7J-K).

Nuclear import of PYDV-P mutants is confirmed by a yeast-based assay

To confirm the *in planta* localization data for PYDV-P NLS and mutants thereof, a yeast-based nuclear import assay (NIA) was employed (Figure 3.8). In this assay, only proteins containing a functional NLS will facilitate the nuclear import of a transcriptional activator required for expression of a reporter gene in yeast cells (Zaltsman *et al.*, 2007). Consistent with the *in planta* importin BiFC data from Figure 3.7, only the ^{KK36AA} mutant of the 1-41 aa fragment was non-nuclear in the NIA. The 1-41 aa fragment and the remaining mutants, which interacted with Atimportin- α 7 in the nucleus *in planta* (Figure 3.7), were positive in the NIA (Figure 3.8).

DISCUSSION

Determining subcellular localization of sequenced proteins is most commonly based on the use of predictive algorithms. However, prediction algorithms primarily look for basic, arginine/lysine-rich regions, typical of proteins imported into the nucleus using importin- α/β pathways. Many functional, non-canonical NLSs have been identified that do not match the consensus sequence for classical NLSs (Mattaj and Englmeier, 1998; Kumar and Raghava, 2009; Anderson *et al.*, 2012). Consequently, the number of proteins with expected nuclear localization is likely far less than the actual number of nuclear-targeted proteins.

In the present study we have characterized the NLS of PYDV phosphoprotein. Although *in silico* methods predicted a classical monopartite NLS in PYDV-P, our *in vivo* analysis indicates that three separate regions are required for nuclear localization of GFP:PYDV-P fusions. Localization of the GFP:fragment fusions was consistent with the *in silico* prediction, as only the fragment containing the predicted NLS was localized exclusively to the nucleus (Figure 3.2B). As all the fragment:GFP fusions are below the estimated diffusion size limit for nuclear pores (~40 kDa), it is not unusual to see some localization in the nucleus, however as the N-terminal fragment is exclusively nuclear it suggests the presence of an NLS. Alanine-scanning mutagenesis with double mutants identified the amino acids ⁶ISPSRKLRDR¹⁵, ¹⁸SK¹⁹, and ³⁶KK³⁷ as being essential for proper localization of PYDV-P. This appears to be a non-canonical tripartite NLS, as mutations in any of the three regions results in loss of nuclear localization. This can be distinguished from three individual monopartite NLSs, where a mutation in one NLS would not impact the function of the others, and the protein would still be localized to the nucleus. Ultimately, the amino acids determined to be responsible for nuclear import of PYDV-P are ⁶ISPSRKLRDR¹⁵, ¹⁸SK¹⁹, and ³⁶KK³⁷. Attempts to refine the mapped NLS with single amino acid substitutions did not identify any single critical residues essential for nuclear import. This could be due to a greater perturbation of NLS function in the double mutants compared to the single mutations, which would allow PYDV-P to retain the ability to bind the import receptor. As with any studies involving alteration of a protein, it is a possibility that introducing mutations has led to misfolding which is the cause of the differing localization patterns, rather than

the disruption of an NLS. Our data suggests that this is not the case, however. All of the mutants tested in this study were shown to interact with at least one other protein, which suggests that the original structure is being preserved. Additionally, we have observed consistent results across plant and yeast experiments, which suggests that an NLS is being disrupted, and not simply degradation or misfolding of the expressed proteins.

Interestingly, PYDV-P was not found to interact with any of the known *N. benthamiana* importin family members, but did interact with two importin- α isoforms of *A. thaliana*. All site-directed mutants of PYDV-P interacted with *Atimportin- α 7* and *- α 9* except for ^{KK}36^{AA}, which suggests these two amino acids play an important role in binding the nuclear import receptor for PYDV-P. This is consistent with the yeast NIA, the results of which indicated that only the ^{KK}36^{AA} mutant was not imported into the nucleus. Since *N. benthamiana*, but not *A. thaliana*, is a host for PYDV, this may suggest that existence of more than two importin- α isoforms in *N. benthamiana*. As *NbImportin- α 1* shows some sequence homology with *AtImportin- α 1*, future work could attempt to amplify additional importins from *N. benthamiana* based on the sequences of *A. thaliana* importin- α 7 and *- α 9*. Mutations in the other two regions that were shown to alter localization of PYDV-P (amino acids 6-15 and 18-19) interacted with *Atimportin- α 9*, but the localization appeared to be completely non-nuclear. Therefore, it is likely that amino acids ⁶ISPSRKLRDR¹⁵ and ¹⁸SK¹⁹ are not involved in binding *Atimportin- α 7* or

- α 9, but somehow are preventing the P:importin- α 9 complex from being imported into the nucleus.

One explanation for localization and interaction data from mutants ⁶ISPSRKLRDR¹⁵ and ¹⁸SK¹⁹ could be the presence of a nuclear export signal in PYDV-P. In fact, it has been shown previously that the related nucleorhabdovirus *Sonchus yellow net virus* (SYNV) P protein is localized to the nucleus as a GFP fusion, but appears to have nuclear export signals and interacts with the nuclear export receptor Xpo1 (A.O. Jackson and M.M. Goodin, unpublished). Therefore, it is a possibility that only amino acids ^{KK}36^{AA} are required for nuclear import and importin- α binding, and mutation of ⁶ISPSRKLRDR¹⁵ and ¹⁸SK¹⁹ could be causing or preventing a structural change that exposes an NES or masks the NLS. Some possible evidence for the presence of an NES in PYDV-P can be seen in Figure 3.2E - although the PYDV-P Fragment D:GFP fusion is smaller than the diffusion limit for nuclear pores (33 kDa vs. ~40 kDa), no GFP is observed inside the nucleus. This could indicate the presence of an NES in this fragment, although this has not yet been investigated. Shuttling of the PYDV-P protein between the nucleus and the cytoplasm makes biological sense when compared to similar rhabdoviruses. SYNV-P has been shown to interfere with changes in host gene expression induced by SYNV-M, but only when SYNV-P is localized to the cytoplasm (Jackson *et al.*, 2005). Furthermore, the P protein of rhabdoviruses is involved in the viral replication complex, and the P proteins of other plant rhabdoviruses have been implicated as suppressors of host antiviral defense (Johansen and Carrington, 2001; Jackson *et al.*, 2005). Additionally, it has been shown that SYNV-N can re-localize SYNV-P to the

nucleus (Jackson *et al.*, 2005), and in this study, that PYDV-N can re-localize NLS-deficient P mutants to the nucleus. This provides a method by which some PYDV-P protein could function in the cytoplasm while interaction with PYDV-N could retain some P protein for replication and transcription.

The NLS of PYDV-P was predicted to be a classical monopartite NLS, however it seems to more closely match a non-canonical NLS identified in the human Cdc6 protein (Takei *et al.*, 1999). A consensus sequence for the Cdc6 NLS was determined to be (S/T)PXKR(L/I), and is conserved among humans, fungi, and *Xenopus* (Takei *et al.*, 1999). The corresponding sequence in the *Arabidopsis thaliana* homologue of Cdc6 (Genbank accession NM 179806.1), ³⁴SRKRKL³⁹, does not exactly match the conserved NLS sequence; an arginine is substituted for the proline, and the position of the lysine and arginine are reversed. However, this more closely resembles the sequence in PYDV-P, which also has the positions of the lysine and arginine reversed. Cdc6 is localized to the nucleus at steady-state but, similar to PYDV-P, does not interact with importin- α and its current method of import is unknown (Takei *et al.*, 1999), which suggests that proteins containing this NLS are transported into the nucleus via a novel pathway.

Phosphorylation of cargo proteins is emerging as an important step in trafficking between the cytoplasm and nucleus, as phosphorylation of different cargos has been shown to either enhance or inhibit nuclear import in some cases (Nardozzi *et al.*, 2010). Phosphorylation has been shown to enhance nuclear import by increasing affinity for import receptors or docking at nuclear pores, or by inducing

conformational changes leading to the exposure of an NLS or the disruption of an NES (Nardozzi *et al.*, 2010). Cdc6 is a protein essential for DNA replication, and is localized to the nucleus during G1 phase of cell replication, but is found in the cytoplasm during S and G2 phases (Saha *et al.*, 1998). Furthermore, studies of Cdc6 in yeast determined that the change in localization from nucleus to cytoplasm is due to phosphorylation of several serine residues (Delmolino *et al.*, 2001, Takei *et al.*, 1999). As a predicted phosphoprotein, it is likely that phosphorylation plays an important role in the function of PYDV-P. This could explain why mutation of several serines in the PYDV-P NLS (^{S7A}, ^{S9A}, ^{S18A}), which are common targets for phosphorylation, lead to a loss of nuclear localization.

Comparison of the PYDV NLS to the predicted NLSs of other sequenced nucleorhabdoviral P proteins shows a lack of conservation among these NLSs (Table 3.1). Several viruses have no predicted NLSs, and the viruses that do have predicted NLSs are located in the C-terminus of the protein, in contrast to the NLS identified in PYDV-P. A BLAST analysis of the predicted P NLSs did not identify any similar sequences in the other Nucleorhabdoviral proteins.

Our BiFC data suggests that the N:P interaction occurs before nuclear import occurs, which is consistent with previous studies (Anderson *et al.*, 2012). Whereas non-nuclear mutants of PYDV-N could inhibit the nuclear import of PYDV-P (Anderson *et al.*, 2012), it appears that non-nuclear mutants of PYDV-P can be ferried into the nucleus by PYDV-N, suggesting a mechanism by which PYDV-P could be retained in the nucleus – the importance of which is discussed above. This could

be due to a N:P interaction domain that overlaps with the P-NLS, as the functioning NLS of PYDV-N can override the non-functioning NLS of PYDV-P when interaction occurs. The N:P interacting domain has previously been determined to reside in fA of PYDV-P. Only one mutant was shown to interact with PYDV-N outside the nucleus, ^{AR22AA} (Figure 3.5M). It is possible that this mutation could be interfering with proper interaction with PYDV-N as discussed above. Alternatively, this region of PYDV-P (^{22ARSAPYDPVKQAKYKK³⁷}) almost matches the consensus sequence for a classical bipartite NLS (KRX₁₀₋₁₂K[KR]X[KR][KR]; Kosugi *et al.*, 2009). If the PYDV-P NLS is indeed a bipartite NLS, amino acids ^{22AR²³} may interact with the minor groove of importin- α , and may be more important for nuclear import during N:P interaction.

In plants, there are multiple pathways a cargo protein can use to enter the nucleus, some of which include phosphorylation or conformational change exposing an NLS; heterocomplex formation resulting in ‘piggybacking’ into the nucleus; and cytoplasmic or nuclear retention due to protein:protein interactions (Meier and Sommers, 2011). Further complicating identification of import pathways is the fact that none of these groups are mutually exclusive, as we have demonstrated in this study. Taken together, this emphasizes the importance of characterizing NLSs and import pathways in order to more accurately predict the sub-cellular localization of viral proteins, and understand how virus are transported throughout the cell.

TABLE 3.1. Predicted nuclear localization signals in phosphoproteins of nucleorhabdoviruses.

Only the PYDV signal has been mapped physically, others have been predicted using *in silico* methods. The numbers indicate the amino acid residues in the full-length proteins that mark the predicted NLSs. MFSV, maize fine streak virus; SYNV, sonchus yellow net virus; RYSV, rice yellow stunt virus; MMV, maize mosaic virus; IMMV, Iranian maize mosaic virus; TaroVCV, taro vein chlorosis virus.

Virus	Genbank Accession	Type	Predicted NLS
MFSV	NC_005974.1	none	n/a
SYNV	KF264454.1	none	n/a
RYSV	NC_003746.1	Bipartite	²⁶⁵ RKDSHHYRTVVSRIEKK ²⁸¹
MMV	NC_005975.1	Dual monopartite	²⁶³ KRPR ²⁶⁶ , ¹⁵⁴ PSIKRKA ¹⁶⁰
IMMV	NC_011542.1	Dual monopartite	²⁶⁵ KKPR ²⁶⁸ , ¹⁵⁶ PNPKRKH ¹⁶²
TaroVCV	NC_006942.1	none	n/a
PYDV	NC_016136.1	Monopartite	⁸ PSRKLRD ¹⁴

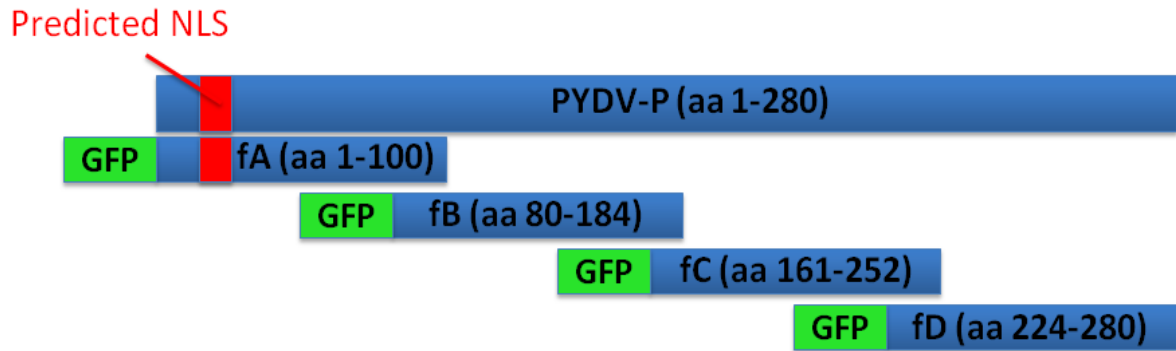


FIGURE 3.1. Size and position of deletion fragments of PYDV-P.

Four overlapping fragments were created from the 280 amino acid PYDV-P protein. fA is the N-terminal 100 amino acids of PYDV-P, and is expected to have a molecular mass of 38 kDa. fB spans amino acids 80-184 of PYDV-P, and has a predicted mass of 38 kDa. fC corresponds to amino acids 161-252 of PYDV-P, and the mass is predicted to be 37 kDa. The C-terminal fragment fD (amino acids 224-280) is the smallest at a predicted mass of 33 kDa. The predicted NLS is indicated as a red band.

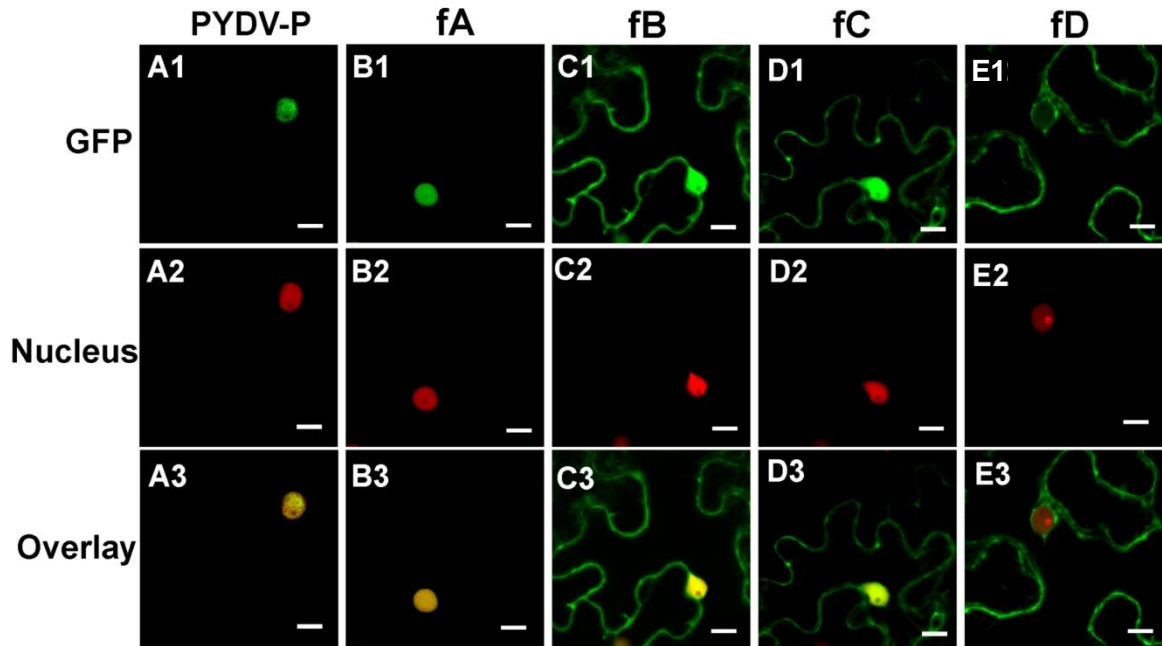


FIGURE 3.2. Confocal micrographs showing localization of GFP:protein fusions expressed transiently in transgenic *N. benthamiana* plants expressing a RFP-ER marker.

Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP-H2b (a nuclear marker), and the resultant overlay, respectively. **(A1-3)** Localization of GFP:P fusion. **(B1-3)** Amino acids 1-100 of PYDV-P. **(C1-3)** Amino acids 80-184 of PYDV-P. **(D1-3)** Amino acids 161-252 of PYDV-P. **(E1-3)** Amino acids 224-280 of PYDV-P.

Scale bar = 10 μ m.

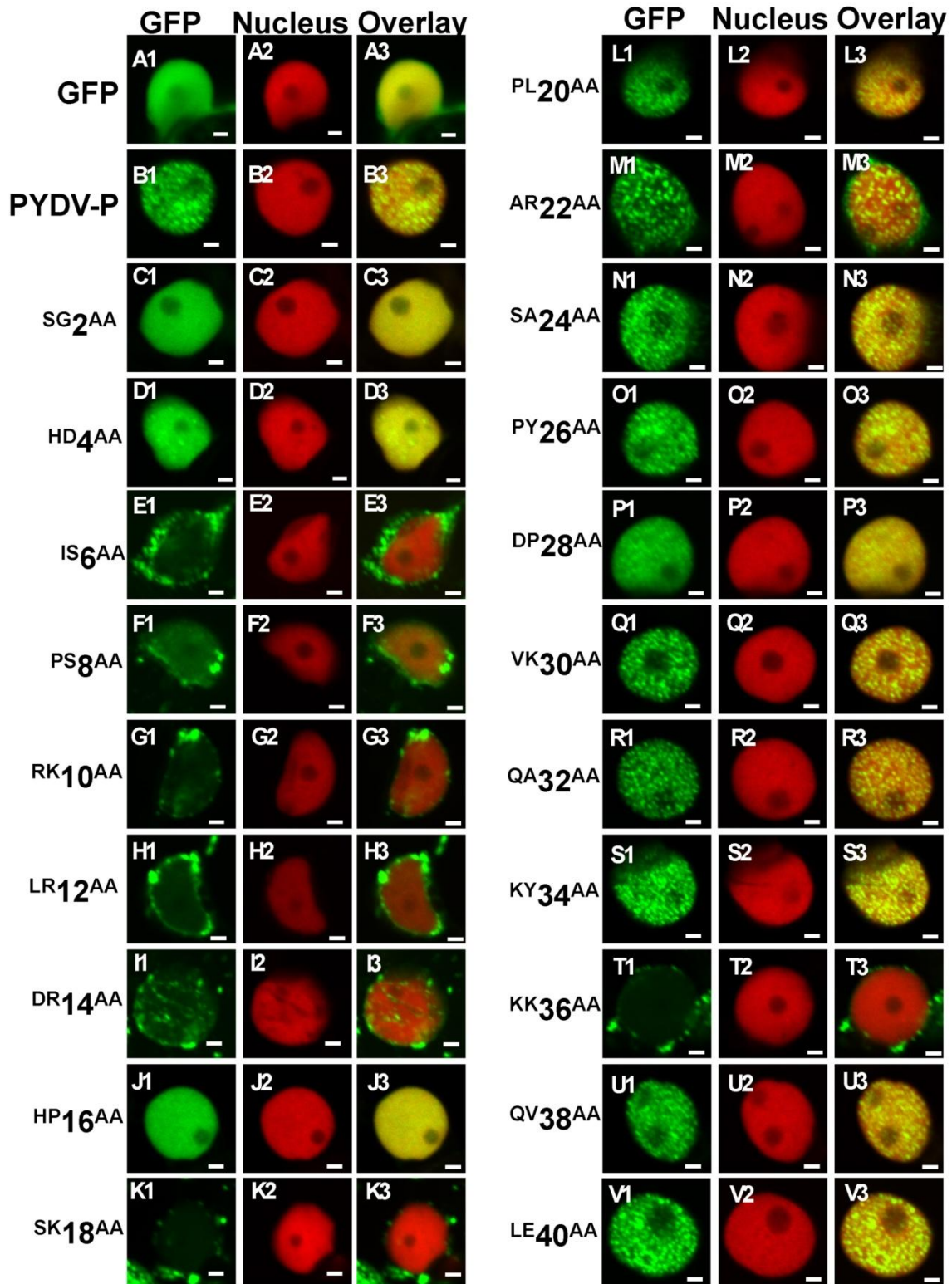


FIGURE 3.3. Single plane confocal micrographs of PYDV-P and site-directed mutants fused to GFP, expressed in transgenic *N. benthamiana* expressing an RFP-H2b marker.

Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP-H2b (nuclear marker), and the resultant overlay, respectively. **(A1-3)** GFP only. **(B1-3)** GFP:PYDV-P fusion. **(C-V)** PYDV-P site-directed double mutants. Numbers indicate the amino acid residue in the full-length P protein where two residues were changed to alanine. Scale bar = 2 μ m.

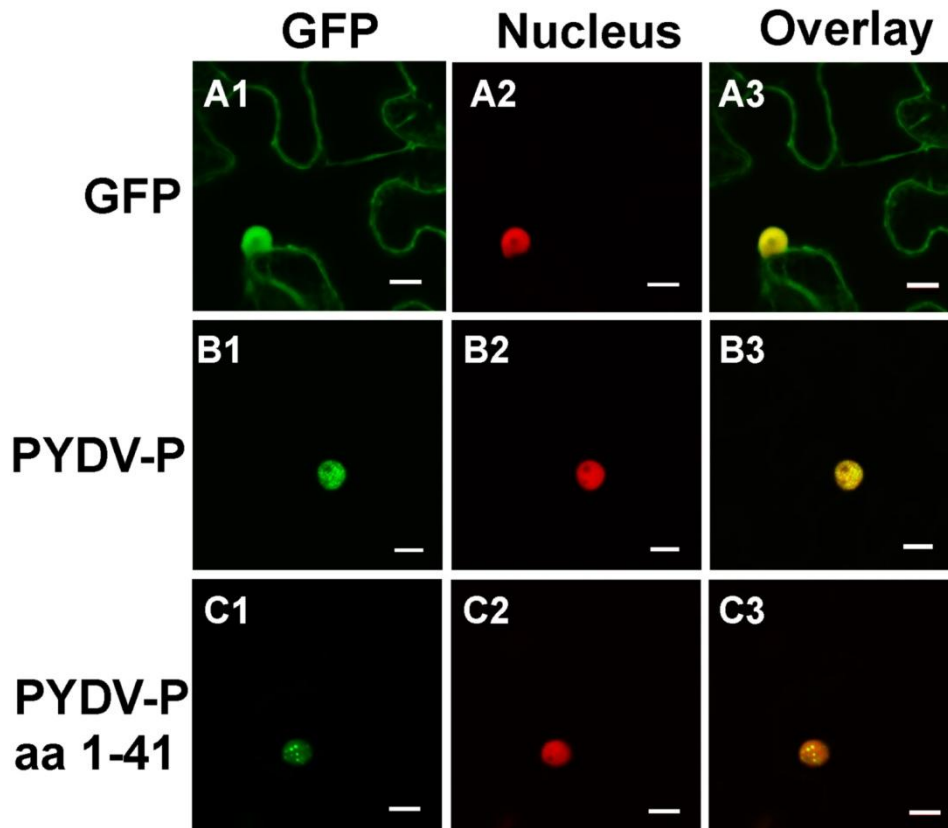


FIGURE 3.4. Single-plane confocal micrographs of GFP:PYDV-P fusions and amino acids 1-41 of PYDV-P expressed in transgenic *N. benthamiana* expressing RFP fused to a nuclear marker, histone 2B.

Shown in panels 1, 2, and 3 are micrographs of GFP-fusions, RFP-nuclei, and the resultant overlay, respectively. **(A1-3)** GFP only. **(B1-3)** GFP:PYDV-P fusion. **(C1-3)**

Amino acids 1-41 of PYDV-P fused to GFP. Scale bar = 10 μ m.

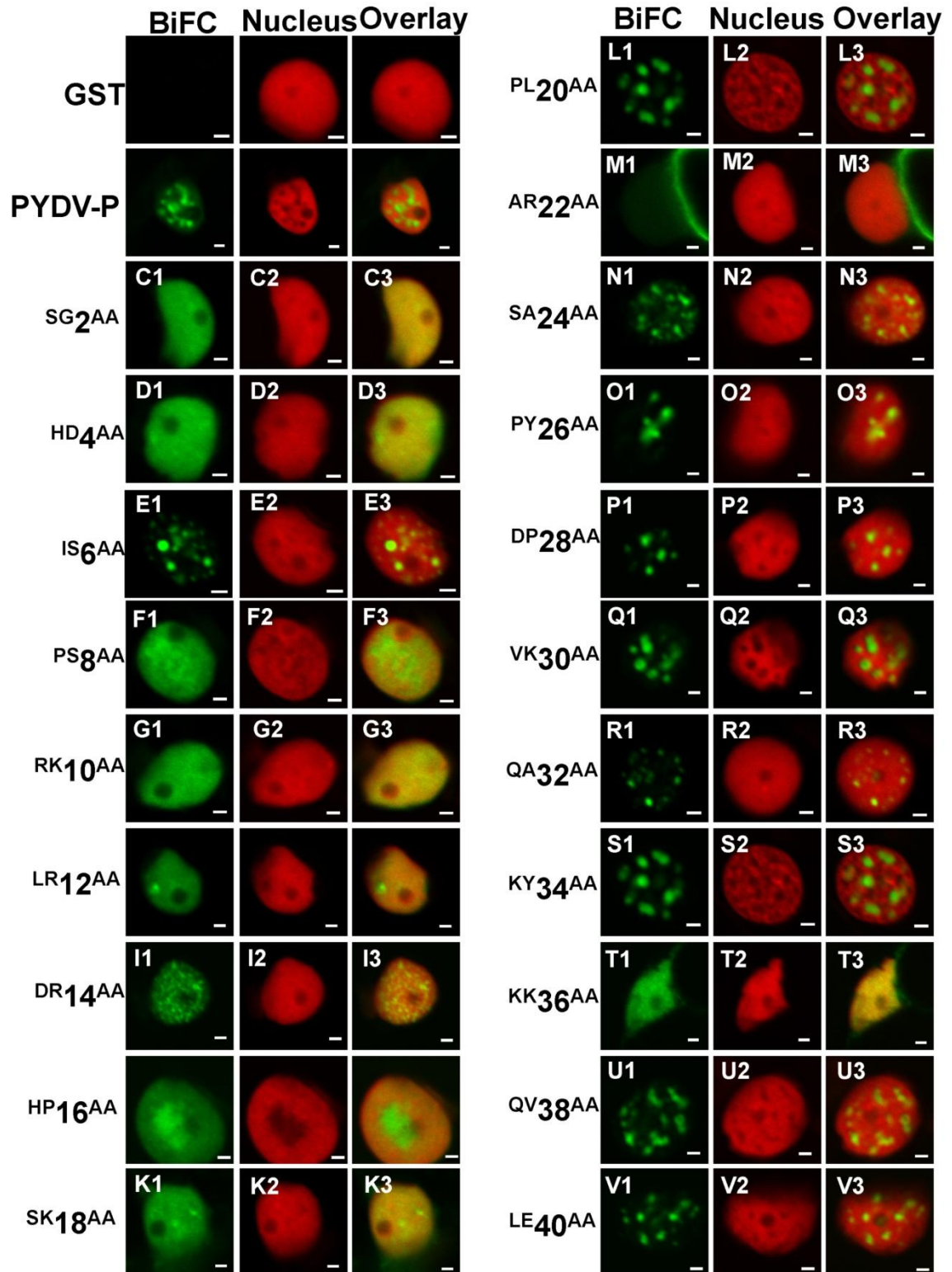


FIGURE 3.5. Confocal micrographs showing PYDV-P mutant protein interactions with PYDV-N determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-N expressed as a fusion to the carboxy-terminus of YFP. **(A1-3)** BiFC between GST and PYDV-N. **(B1-3)** BiFC between PYDV-P and PYDV-N. **(C-L)** BiFC between PYDV-N and double mutants of PYDV-P described in Figure 3.3. Scale bar = 2 μ m.

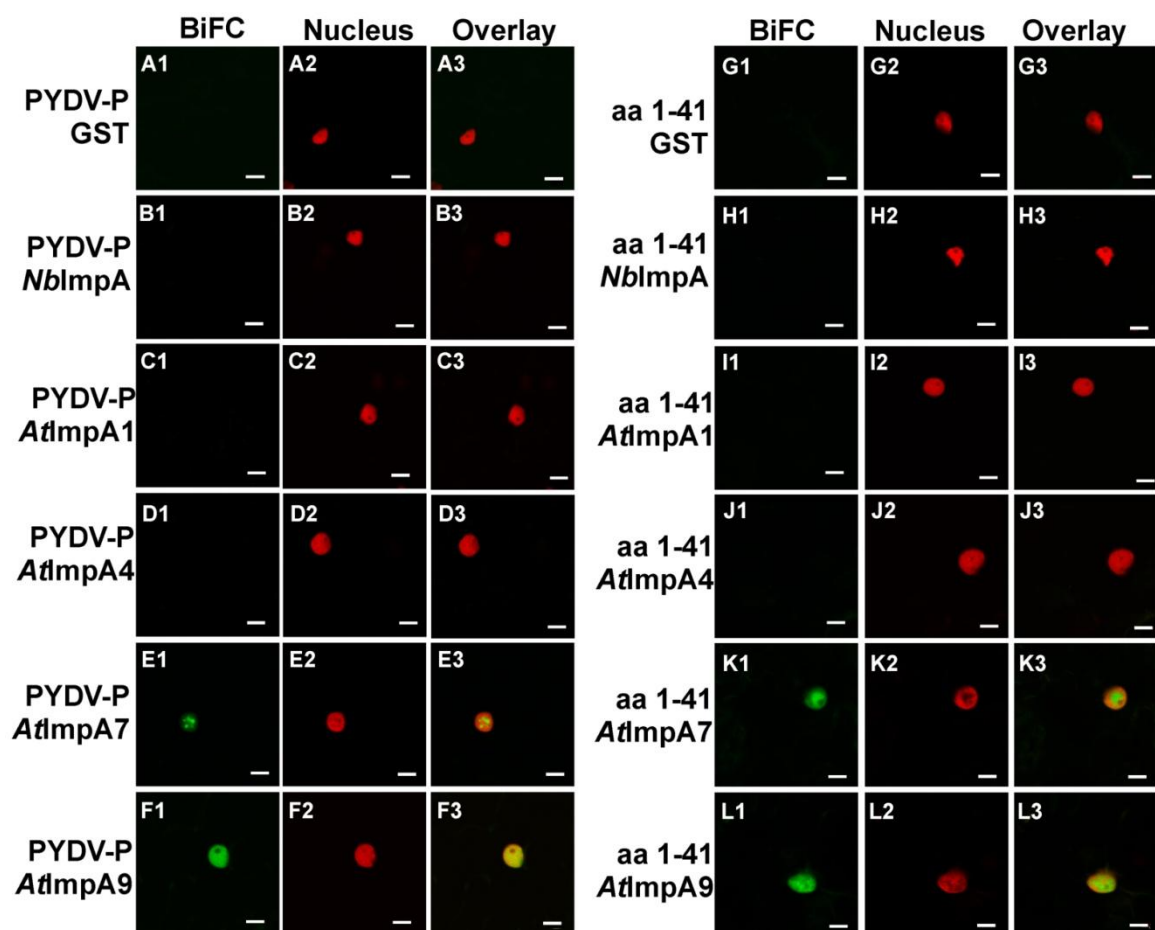


FIGURE 3.6. Confocal micrographs showing PYDV-P and amino acids 1-41 of PYDV-P interact with importin- α isoforms determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against *N. benthamiana* (*Nb*) or *A. thaliana* (*At*) importin- α isoforms expressed as a fusion to the carboxy-terminal of YFP. **(A, G)** BiFC of GST with PYDV-P or amino acids 1-41 of PYDV-P, respectively. **(B, H)** Representative micrograph of BiFC between PYDV-P or amino acids 1-41 of PYDV-P and *Nb*importin- α 1, - α 2, or - β . **(C, I)** BiFC between *At*importin- α 1 and PYDV-P or amino acids 1-41 of PYDV-P, respectively. **(D, J)** BiFC between *At*importin- α 4 and PYDV-P or amino acids 1-41 of PYDV-P, respectively. **(E, K)** BiFC between *At*importin- α 7 and PYDV-P or amino acids 1-41 of PYDV-P, respectively. **(F, L)** BiFC between *At*importin- α 9 and PYDV-P or amino acids 1-41 of PYDV-P, respectively. Scale bar = 10 μ m.

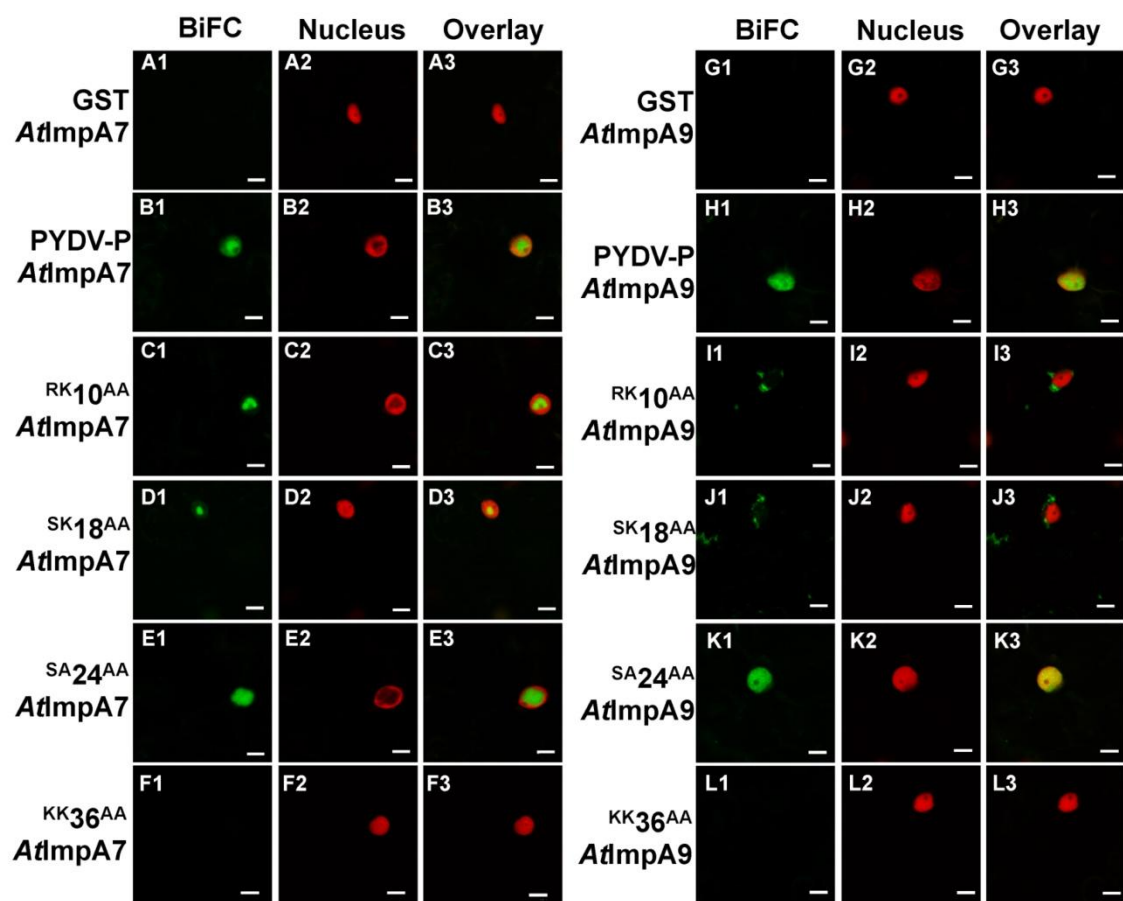


FIGURE 3.7. Confocal micrographs showing results of BiFC assay to determine interaction between site-directed PYDV-P mutants and *Atimportin-α7* or *-α9*.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing fluorescent nuclear marker protein fusions. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. **(A, G)** BiFC between GST and *Atimportin-α7* or *α9*, respectively. **(B, H)** Interaction between PYDV-P and *Atimportin-α7* or *α9*, respectively. **(C-F)** BiFC between importin-*α7* and double mutants of PYDV-P. **(I-L)** BiFC between importin-*α9* and double mutants of PYDV-P. **(C/I)** is representative of BiFC between *Atimportin-α7/9* and double mutants of PYDV-P between amino acids 6-15. **(E/K)** is representative of the BiFC between *Atimportin-α7/9* all remaining site-directed mutants. Scale bar = 10 μ m.

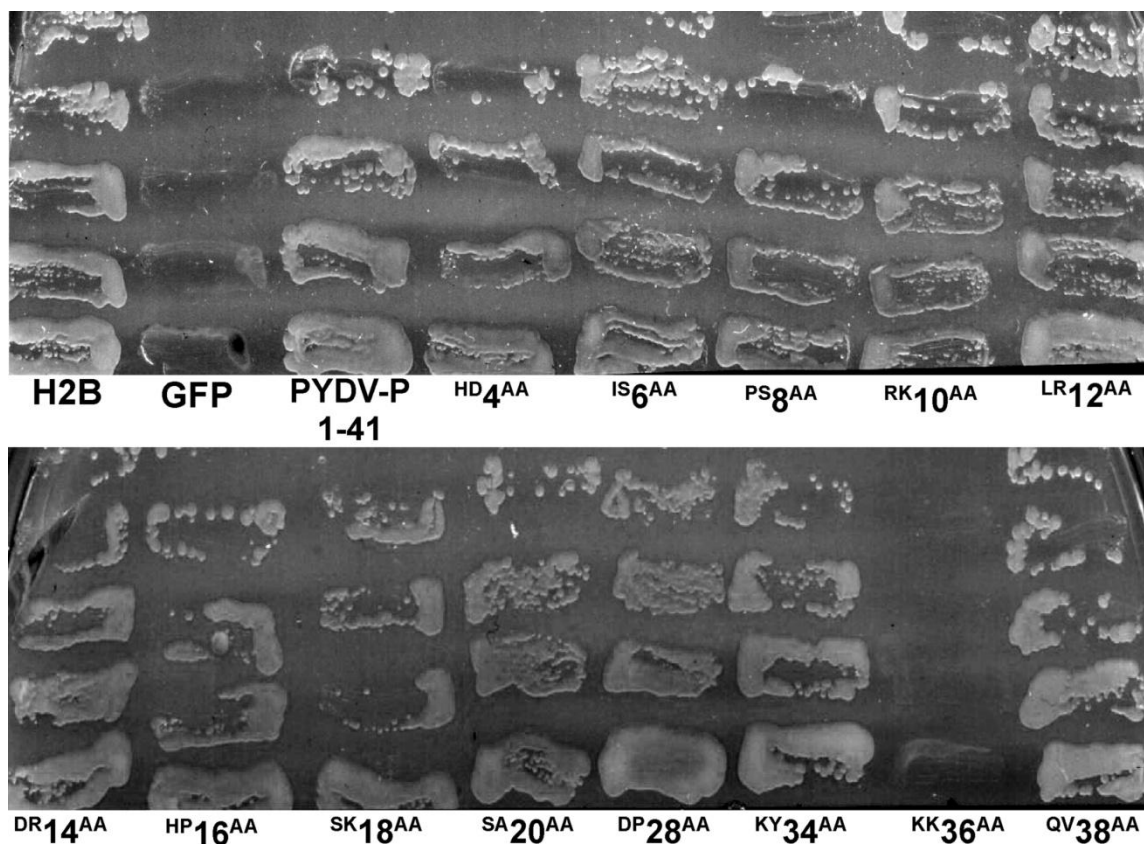


FIGURE 3.8. Yeast-based assay for identification of proteins containing a functional NLS.

Positive [histone 2b (H2B)] and negative (GFP) control proteins, along with amino acids 1-41 of PYDV-P and mutants thereof, were expressed from pNIA-DEST in yeast strain L40. Only proteins containing a functional NLS are able to facilitate yeast growth on media lacking histidine, and were scored as positive in this assay.

CHAPTER IV

Nuclear import and membrane association of *Potato yellow dwarf virus* matrix protein is mediated by overlapping motifs

Members of the family Rhabdoviridae are minus-sense, single-stranded RNA viruses containing members able to infect humans, animals, fish, insects, and plants (Kuzmin *et al.*, 2009). The plant-adapted members are assigned to two genera based on their sites of replication and morphogenesis. *Potato yellow dwarf virus* (PYDV) is the type species of the *Nucleorhabdovirus* genus, the members of which replicate in nuclei of infected cells (Jackson *et al.*, 2005). As minus-sense RNA viruses, the genomic RNA alone is not infectious. Instead, the minimal infectious unit consists of genomic RNA encapsidated over its entire length by a nucleocapsid (N) protein, along with a polymerase (L) and a phosphoprotein (P; Goodin *et al.*, 2001; Jackson *et al.*, 2005; Ammar *et al.*, 2009). The matrix (M) protein of PYDV is critical for the successful infection and spread of PYDV. During morphogenesis, the nucleocapsid is condensed into viral cores by the matrix (M) protein, which then bud through the inner nuclear membrane, acquiring a host-derived envelope embedded with glycoprotein (G), and into the perinuclear space (Goodin *et al.*, 2001, 2007; Martin *et al.*, 2009). We have previously shown that the M protein of a related *Nucleorhabdovirus*, *Sonchus yellow net virus* (SYNV), is likely involved in the nuclear export of viral nucleocapsids and interacts with SYN-4 (homologue to PYDV-M) in cell-to-cell movement (Min *et al.*, 2010).

We have also shown previously that expression of PYDV-M is sufficient to induce the intranuclear accumulation of the inner nuclear membranes

(Bandyopadhyay *et al.*, 2010). The biological relevance of this is obvious, as PYDV is known to bud through the inner nuclear membrane to acquire a lipid envelope. Additionally, host membranes are common targets for viruses for the formation of replication centers. As replication and morphogenesis of PYDV occurs inside the nucleus, in order for infection to be successful, the PYDV-M protein must contain a functional nuclear localization signal (NLS) that directs this protein to the nucleus. While NLSs have been identified or predicted in the N proteins of several Nucleorhabdoviruses (Anderson *et al.*, 2012; Goodin *et al.*, 2001), the available data for identified NLSs in Nucleorhabdovirus matrix proteins is lacking.

We have shown previously that PYDV-M is capable of directing Green fluorescent protein (GFP) exclusively to the nucleus when expressed in plant cells as a GFP:PYDV-M fusions (Ghosh *et al.*, 2008). In addition, *in silico* prediction (pSORTII; Horton and Nakai, 1997) has identified a possible NLS in PYDV-M. However, it is important to confirm that the predicted NLS is functional, as we have identified proteins with predicted NLSs that are not localized to the nucleus under steady state observation (Min *et al.*, 2010). In addition, many NLSs have been identified and characterized that are not detected using predictive algorithms (Mattaj and Englmeier, 1998; Kumar and Raghava, 2009; Anderson *et al.*, 2012).

In this study, we report the characterization of the NLS responsible for nuclear targeting of the matrix protein of *Potato yellow dwarf virus*. We have previously shown that PYDV-M interacts with PYDV-N, -M, -Y, and -G (Bandyopadhyay *et al.*, 2010). This raises the possibility that interaction with other

PYDV proteins may play a role in facilitating nuclear transport of M. These studies aim to improve predictive algorithms and understanding of plant-virus interactions by characterizing the NLSs of these proteins and their impact on sub-cellular localization and interactions.

METHODS

Plant materials and virus maintenance

All plants, including transgenic marker lines, were maintained in greenhouse on open benches under ambient conditions (Martin *et al.*, 2009). PYDV (American Type Culture Collection accession PV-234) was maintained by serial passage in *Nicotiana benthamiana* and *Nicotiana rustica* in insect-proof cages under ambient conditions.

Generation of site-directed mutants

Site-directed mutagenesis was performed using the Change-IT™ Multiple Mutation Site-Directed Mutagenesis Kit (USB) according to protocols provided by the manufacturer. All mutagenesis was conducted using sequence-validated portions of the PYDV-P and PYDV-M open reading frames cloned into plasmid pDONR221 (Invitrogen). Mutagenized clones were validated by DNA sequencing to confirm the presence of specific mutations.

Protein expression in plant cells

Expression of autofluorescent protein fusions in plant cells for localization and bimolecular fluorescence complementation (BiFC) assays were performed as

described previously (Chakrabarty *et al.*, 2007; Goodin *et al.*, 2007; Martin *et al.*, 2009; Anderson *et al.*, 2012). In this study, the vector pSITE-2C (GFP-fusion) was used for localization assays, and pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays, respectively. Recombinant vectors were transformed into *A. tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted as described previously (Goodin *et al.*, 2002). Each expression construct was examined in 1 cm² leaf pieces taken from a minimum of two leaves, from a minimum of two independent plants. Multiple micrographs representative of several hundred examined cells were acquired for documentation.

Laser scanning confocal microscopy

All microscopy was performed on an Olympus FV1000 laser-scanning confocal microscope as described previously (Goodin *et al.*, 2005). Micrographs for dual-color image were acquired sequentially, as described in Goodin *et al.* (2007).

Nuclear import assay in yeast

Nuclear import assays were conducted as described previously (Bandyopadhyay *et al.*, 2010). Recombinant pNIA-DEST plasmids were transformed into *Saccharomyces cerevisiae* strain L40 (Zaltsman *et al.*, 2007). Transformed yeast were grown at 30°C for 3 days, on minimal media lacking tryptophan (Trp-). Colonies were then re-streaked onto minimal media lacking both tryptophan and histidine (His-), containing 50 mM 3-amino-1,2,4-triazole (3AT). Growth of yeast cultures on Trp-/His- media indicates a functional NLS in proteins expressed from pNIA-DEST (Zaltsman *et al.*, 2007).

RESULTS

An NLS is present in the C-terminus of PYDV-M

We have previously shown that GFP-fusions of PYDV-M localize exclusively to the nucleus when expressed in transgenic *N. benthamiana* (Bandyopadhyay *et al.*, 2010). In addition, prediction algorithms have identified a possible bipartite NLS between amino acids 213-229 of PYDV-M (Horton and Nakai, 1997).

To determine if this region of PYDV-M is responsible for the nuclear localization of this protein, we created three overlapping fragments of PYDV-M as GFP fusions (Figure 4.1), and determined the subcellular localization *in planta*. Fragments 1 and 2 were excluded from the nucleus (Figure 4.2B-C), whereas the C-terminal Fragment 3 (aa 150-253) was the only fragment observed to induce intranuclear accumulations (Figure 4.2D), suggesting an NLS may be present in this fragment. Two overlapping fragments of PYDV-M Fragment 3 were then created and expressed as GFP-fusions *in planta*. Neither Fragment 4 (aa 150-224) nor Fragment 5 (aa 199-253) were localized to the nucleus, despite the predicted NLS being contained within Fragment 5 (Figure 4.2E, F). In order to further define the location of the NLS in PYDV-M, alanine-scanning mutagenesis was performed in the context of full length M protein (Figure 4.3). These mutants, which span the predicted NLS, demonstrated the amino acids critical for nuclear import. Scanning mutagenesis upstream or downstream of this region did not influence nuclear localization. Despite being a predicted bipartite NLS, only two amino acids (²²⁵KR²²⁶) appeared to be required for NLS function (Figure 4.3J), whereas the other basic region and separating amino acids could be mutated without affecting nuclear-

localization (Figure 4.3C-I). Fine mapping using single amino acid mutations revealed that the individual amino acids K²²⁵ and R²²⁶ are required for NLS function (Figure 4.3N-O).

The predicted NLS region of PYDV-M is non-nuclear as a GFP-fusion at steady state, but interacts with importin- α

In order to test function of the PYDV-M NLS outside the context of the full-length protein, aa 203-236 of PYDV-M were expressed as a GFP-fusion in plant cells. Expression of GFP alone in plant cells results in localization throughout the cell, including the nucleus (Figure 4.4A). However, the addition of aa 203-236 of PYDV-M to GFP resulted in exclusion from the nucleus at steady state (Figure 4.4B). Since the 203-236:GFP fusion is smaller than the exclusion limit for nuclear pores (30.7 kDa compared to ~40 kDa exclusion limit), some localization in the nucleus by diffusion would be expected unless the fusion protein is being actively exported from the nucleus. However, when BiFC was performed between PYDV-M aa 203-236 and *Nb*Importin- α 1, they were shown to interact, and the interaction was localized to the nucleus (Figure 4.5C). This suggests the possibility of an NLS and a nuclear export signal (NES) in amino acids 203-236 of PYDV-M. Unfortunately, consensus NES sequences are poorly defined, and an NES sequence was not detected in PYDV-M by any prediction algorithms. Alanine-scanning mutagenesis of the 203-236:GFP fusion did not identify a region able to restore nuclear localization *in planta* (Figure 4.4C-L).

PYDV-M mutant cannot be ferried into the nucleus by the PYDV Movement protein (Y), Glycoprotein (G), or wild-type Matrix protein (M).

It has previously been shown that PYDV-M interacts with itself, PYDV-N, PYDV-Y, and PYDV-G (Bandyopadhyay *et al.*, 2010). These interactions could occur prior to or following nuclear import of PYDV-M. To address the possibility that PYDV-M enters the nucleus as homo- or hetero-oligomeric complexes with PYDV-Y, -G, or -N, bimolecular fluorescence complementation assays were performed using the PYDV-M mutants.

Assays with PYDV-M showed that all mutants retained their ability to self-interact (Figure 4.6C-L). As expected, interaction with the ²²⁵KR²²⁶ mutant, which was no longer localized to the nucleus as a GFP-fusion, occurred exclusively outside the nucleus (Figure 4.6J). However, contrary to expectation, several of the mutants which were localized to the nucleus as GFP-fusions, only interacted with PYDV-M outside of the nucleus (Figure 4.6K-L).

Assays with PYDV-Y showed that three of the PYDV-M mutants (^{KK}213^{AA}, ^{SD}217^{AA}, and ^{LL}223^{AA}) fails to interact with PYDV-Y (Figure 4.7D, F, I). Similar to interaction with PYDV-M, mutations introduced between amino acids 221-229 resulted in interaction occurring primarily outside of the nucleus (Figure 4.7H-K). In a similar fashion to interaction with PYDV-M, interaction of PYDV-Y with the PYDV-M ^{KR}225^{AA} mutant, which is not localized to the nucleus as a GFP-fusion, occurred exclusively outside the nucleus (Figure 4.7J).

BiFC assays with PYDV-G showed that the same three mutants (^{KK}213^{AA}, ^{SD}217^{AA}, and ^{LL}223^{AA}) do not interact with PYDV-G (Figure 4.8D, F, I). In addition,

mutations introduced between amino acids 221-229 resulted in interaction primarily outside of the nucleus (Figure 4.8H-L), and interaction with the ²²⁵KR²²⁶ mutant was exclusively non-nuclear (Figure 4.8)).

PYDV-N can ferry PYDV-M mutant into the nucleus

It has been shown previously that interaction between PYDV-M and PYDV-N occurs in the nucleus (Bandyopadhyay *et al.*, 2010). BiFC between PYDV-N and PYDV-M mutants showed that the same mutants that no longer interacted with PYDV-Y or -G (^{KK}213^{AA}, ^{SD}217^{AA}, and ^{LL}223^{AA}) no longer interacted with PYDV-N (Figure 4.9D, F, I). Interestingly, the interaction of PYDV-M with the PYDV-M ²²⁵KR²²⁶ mutant, which was non-nuclear as a GFP-fusion, occurred exclusively inside the nucleus (Figure 4.9J). The remaining mutants, that did not affect nuclear import of PYDV-M, showed interaction similar to that seen with wild-type M (Figure 4.9C, E, G-H, K-L). Previous studies have shown that interaction of PYDV-M with a non-nuclear PYDV-N mutant causes the PYDV-N mutant to be re-localized to the nuclear periphery, rather than throughout the cytosol (Anderson *et al.*, 2012). In addition, BiFC between a non-nuclear PYDV-M mutant and a non-nuclear PYDV-N mutant showed interaction that was excluded from the nucleus (data not shown). This suggests that PYDV-N and PYDV-M interact before import into the nucleus, and that this interaction may allow a mutant without a functioning NLS to be recruited to the nucleus.

Interaction of PYDV-M mutants with Importin- α

In the classical nuclear import pathway, NLSs on cargo proteins interact with the nuclear import receptor importin- α , which has two known isoforms in *N. benthamiana* (Kanneganti *et al.*, 2007). We have previously shown that PYDV-N preferentially interacts with *N. benthamiana* importin- α 1 (*Nbimportin- α 1*; Martin *et al.*, 2009), and have identified a non-canonical NLS in PYDV-N that interacts with *Nbimportin- α 1* outside the context of full-length protein (Anderson *et al.*, 2012). Therefore to investigate the role of the predicted NLSs in PYDV-M, we conducted BiFC assays to test interaction of the predicted NLS, outside the context of full-length protein, with *Nbimportin- α 1*. Results of this assay showed that wild-type PYDV-M, along with the predicted NLS of PYDV-M, interacted with *Nbimportin- α 1* inside the nucleus (Figure 4.5B-C). The PYDV-M^{KR225AA} mutant, which was non-nuclear when expressed as a GFP-fusion, no longer interacted with *Nbimportin- α 1* (Figure 4.5K). Conversely, all mutants that were still localized to the nucleus when expressed *in planta* as GFP-fusions interacted with *Nbimportin- α 1* (Figure 4.5D, F-J, L), with the exception of ^{KK213AA} (Figure 4.5E).

Nuclear Import of PYDV-M mutants is confirmed by a yeast-based assay

To confirm the *in planta* localization data for PYDV-M NLS and mutants thereof, a yeast-based nuclear import assay (NIA) was employed (Figure 4.10). In this assay, only proteins containing a functional NLS will facilitate the nuclear import of a transcriptional activator required for expression of a reporter gene in yeast cells (Zaltsman *et al.*, 2007). Consistent with the *in planta* localization pattern,

the ^{KR}225^{AA} mutant of the 203-236 aa fragment was negative in the yeast nuclear import assay. However, the 203-236 aa fragment and all other mutants, which were localized outside the nucleus *in planta* (Figure 4.4), were positive in the NIA (Figure 4.10).

The PYDV-M NLS can be uncoupled from membrane re-localization

We have previously reported the ability of PYDV-M to induce the intranuclear accumulations of the inner nuclear membrane (Bandyopadhyay *et al.*, 2010). While conducting the alanine-scanning mutagenesis across the predicted NLS, we observed that one of the mutants (²²³LL²²⁴) was localized to the nucleus, but did not induce the accumulation of membranes (Figure 4.3I). Fine mapping suggests that substitution of both ²²³LL²²⁴ is necessary to disrupt membrane re-localization, as the single mutants L²²³ and L²²⁴ showed membrane accumulations similar to wild-type PYDV-M (Figure 4.3L-M). In addition, GFP fusions of PYDV-M Fragment 5 expressed *in planta* were excluded from the nucleus, but caused proliferation of membranes in the ER (Figure 4.2F). Introducing the ²²³LL²²⁴ mutation to Fragment 5 resulted in the same localization pattern as wild-type Fragment 5, but did not induce proliferation of ER membranes (Figure 4.11E). Introducing the ²²³LL²²⁴ mutation to Fragment 3 resulted in GFP:protein fusion localized in the nucleus, but with the absence of any intra-nuclear membrane accumulations, (Figure 4.11C, F), although the nuclear import of this fusion is slightly inhibited. This suggests that the nuclear localization and the membrane re-localization activity of PYDV-M can be uncoupled. However, membrane

accumulations are not observed during expression of GFP-fusions of the ²²⁵KR²²⁶ mutant of PYDV-M (Figure 4.3J, N,O), suggesting that these amino acids may also play a role in membrane relocalization.

Amino acids 203-236 of PYDV-M also contains a nuclear export signal

The above results have shown that the predicted NLS of PYDV-M (aa 203-236) interacted with importin- α in the nucleus (Figure 4.5) and was positive for nuclear import in a yeast assay (Figure 4.10), but was completely excluded from the nucleus when expressed as a GFP-fusion (Figure 4.4). To determine if the PYDV-M NLS also contains a nuclear export signal (NES), BiFC was performed between PYDV-M, the PYDV-M NLS, and the *A. thaliana* exportin protein XPO1 (Figure 4.12). The results of this BiFC assay showed that PYDV-M interacted with XPO1 in small punctate loci within the nucleus (Figure 4.12B). In contrast, the NLS of PYDV-M also interacted with XPO1, but the interaction was localized primarily outside the nucleus in large aggregations (Figure 4.12C).

DISCUSSION

Determining subcellular localization of sequenced proteins is most commonly based on the use of predictive algorithms. However, prediction algorithms primarily look for basic, arginine/lysine-rich regions, typical of proteins imported into the nucleus using importin- α/β pathways. Many functional, non-canonical NLSs have been identified that do not match the consensus sequence for classical NLSs (Mattaj and Englmeier, 1998; Kumar and Raghava, 2009; Anderson *et*

al., 2012). Consequently, the number of proteins with expected nuclear localization is likely far less than the actual number of nuclear-targeted proteins.

In the present study, *in silico* prediction methods identified an NLS in PYDV-M, however our *in vivo* analysis differs from the expected results. A classical bipartite NLS was predicted in PYDV-M spanning from amino acids 213-229, while we identified only K²²⁵ and R²²⁶ as being critical for nuclear import. These results do not mean that two amino acid signal of KR is sufficient to direct a protein to the nucleus. Rather, in the context of PYDV-M, a lysine at position 225 and an arginine at position 226 are critical for proper import of this protein. We have not investigated whether nuclear import activity is retained when other basic amino acids are substituted at these positions. Although the two critical amino acids K²²⁵ and R²²⁶ are not sufficient to direct a protein to the nucleus, our data suggests, as discussed below, that a 34 amino acid fragment of PYDV-M contains both a functioning NLS and NES. Further evidence that K²²⁵ and R²²⁶ are critical for import of PYDV-M came from the BiFC data in this study. The ^{KR225AA} mutant retained its ability to interact with the PYDV proteins -M, -G, and -Y, but these interactions now occurred completely outside the nucleus. This NLS-deficient mutant was also unable to interact with importin- α , and was not able to be imported to the nucleus in the yeast assay. The *in silico* prediction identified the M NLS as a bipartite NLS, which means the amino acids ^{213KK214} should also be required for nuclear import. Our results have shown that this is not the case. The ^{KK213AA} mutant that was generated was localized to the nucleus as a GFP fusion, was nuclear in the yeast assay, interacted with importin- α in the nucleus. These results show the M NLS is

not a bipartite NLS as predicted, but that the amino acids K²²⁵ and R²²⁶ were the only two amino acids critical for nuclear import of PYDV-M.

We also found that substituting amino acids ²²³LL²²⁴ with alanines results in a nuclear-localized mutant that did not cause the invaginations of nuclear membranes typical of PYDV-M. In addition, the C-terminal 55 amino acids expressed as a GFP fusion in plant cells was not localized to the nucleus, but induced proliferation of ER membranes. Introducing the ²²³LL²²⁴ mutation to this fragment resulted in an ER-localized protein that did not induce membrane proliferation. These results show that amino acids ²²³LL²²⁴ of PYDV-M appear to be important for the re-localization of plant membranes, and that interaction with membranes is not necessary for nuclear import of this protein. There is also evidence that amino acids ²²⁵KR²²⁶ may be involved in membrane re-localization, along with their role in nuclear import. Expressing PYDV-M containing the ²²⁵KR²²⁶ mutation results in a non-nuclear localized mutant that does not cause membrane accumulations, similar to the localization pattern observed in Fragment 4 of PYDV-M (aa 150-224). Fragment 4 contains amino acids ²²³LL²²⁴, that were identified as critical for membrane re-localization, however it does not cause accumulation of membranes. It is likely that because ²²³LL²²⁴ are the terminal residues in this fragment, these residues may be in a different conformation than in the context of full-length PYDV-M. Furthermore, BiFC data of the N: ²²⁵KR²²⁶ mutant interaction showed restored nuclear localization, but the interaction was found throughout the nucleus, opposed to the interaction with the other PYDV-M mutants which were found in sub-nuclear loci. This suggests that amino acids ²²³LL²²⁴ are required for inducing invaginations

of host membranes, and amino acids ²²⁵KR²²⁶ may be required for both nuclear import and membrane re-localization.

Comparison of the PYDV-M NLS to the predicted NLSs of other sequenced nucleorhabdoviral -M proteins shows a low amount of similarity (Table 4.1). Similar to PYDV-M, *Maize fine streak virus* (MFSV) matrix protein also contains a predicted bipartite NLS in the C-terminus of the protein, which suggests that this NLS sequence may be utilized in plants. However, this is an *in silico* prediction, and to date only the PYDV-M NLS has been physically mapped. A BLAST analysis of the predicted PYDV-M NLSs did not identify any similar sequences in other nucleorhabdoviral proteins.

Analysis of the N:M interaction suggests that M-NLS mutants retain the ability to interact with PYDV-N, and that this interaction takes place before nuclear import occurs, which is consistent with previous studies (Anderson *et al.*, 2012). We found that wild-type PYDV-N with a functioning NLS is able to restore nuclear localization to a non-nuclear PYDV-M mutant. However, previous studies have shown that PYDV-M with a functioning NLS appears to recruit non-nuclear PYDV-N mutants to what may be nuclear pores, but cannot recruit PYDV-N into the nucleus (Anderson *et al.*, 2012). In addition, NLS-deficient mutants of both PYDV-N and PYDV-M were still able to interact, and this interaction was localized completely outside the nucleus. This suggests that the N:M interaction domain may be overlapping with the PYDV-M NLS, or that interaction with PYDV-N may block access to the PYDV-M NLS. It was also observed that mutants ^{KK}213^{AA}, ^{SD}217^{AA}, and

^{LL223AA} no longer interacted with PYDV-N, -Y, or -G. This could be due to change in protein conformation caused by mutations that interfere with protein-protein interaction. Alternatively, lack of interaction with the ^{LL223AA} mutant could implicate membrane interaction of PYDV-M as required for interaction with PYDV-N, -Y, or -G. As with any studies involving alteration of a protein, it is a possibility that introducing mutations has led to misfolding which is the cause of the differing localization patterns and lack of interaction. Our data suggests that this is not the case, however. All of the mutants tested in this study were shown to interact with at least one other protein, which suggests that the original structure is being preserved. Additionally, we have observed consistent results across plant and yeast experiments, which suggests the interaction domain is being disrupted, and not simply degradation or extensive misfolding of the expressed proteins.

In planta localization of GFP-fusion of amino acids 203-236 of PYDV-M, which contains the predicted NLS, was found to be completely excluded from the nucleus. When GFP is expressed alone in plant cells, some localization is found in the nucleus from simple diffusion, even though no NLS has been identified. As the predicted NLS fragment of PYDV-M is under the expected size limit for diffusion into the nucleus, it would be expected that localization would be observed throughout the cell, including the nucleus, if no localization signal is present. Since the GFP:NLS fragment was completely excluded from the nucleus, it is likely that this is due to the presence of a nuclear export signal (NES), although no NES was identified with predictive algorithms. Additionally, this 203-236 fragment was shown to interact with *Nbimportin-α1*, and this interaction occurred inside the nucleus, which

suggests the presence of a functioning NLS and import of this protein. Furthermore, the 203-236 amino acid NLS fragment was positive in the yeast nuclear import assay, which indicates that this protein is being imported into the nucleus in yeast cells.

Although yeast and *N. benthamiana* encode different numbers of importin- α isoforms, the similar results from BiFC and pNIA assays suggests that this fragment contains a functioning NLS. The localization and interaction data for the NLS fragment appears to be contradictory, but comparison to other NLSs could explain the localization. This is similar to the 40aa M9 sequence of hnRNP A1, which has been shown to have both non-canonical nuclear import and nuclear export activity (Michael *et al.*, 1995). It is likely that the 203-239 amino acid NLS fragment of PYDV-M contains both a NLS and an NES, which is supported by the interaction between PYDV-M or the M-NLS and *Arabidopsis* XPO1. The NLS fragment appears to interact with importin- α during nuclear import, and is then exported from the nucleus which would give the steady-state localization pattern observed in Figure 4.4. Additionally, the import or export activity of the M9 sequence cannot be uncoupled by mutagenesis (Michael *et al.*, 1995). This could be why mutations in the 203-236 fragment of PYDV-M did not disrupt the potential NES and restore nuclear localization. It would be biologically relevant for both an NLS and NES to be present in PYDV-M. Nucleorhabdoviral M proteins are required for the budding of complete viral particles into the perinuclear space (Goodin *et al.*, 2001, 2007), and a study by Min *et al.*, (2010) provided a model of cell-to-cell movement in which SYNVM protein is exported from the nucleus and forms a movement complex on the ER.

This highlights the importance of determining the sequences responsible for nuclear import and export, and these processes are critical for the successful spread of PYDV.

The difference in steady-state localization patterns between GFP-fusions of PYDV-M and amino acids 203-236 of PYDV-M is likely due to the structure of these proteins. It is likely that the change in localization of PYDV-M between nucleus for virion assembly and cytoplasm for cell-to-cell movement is controlled by a conformational change that exposes an NES and masks the NLS. Therefore, although the 203-236 fragment contains both an NLS and NES, the NLS may be more prominent in the wild-type protein, and the NES more prominent in the 203-236 fragment. Additionally, this adds significance to our N:M BiFC data. As PYDV-N is able to re-localize and retain non-nuclear M mutants in the nucleus, this provides a method by which some M protein could be retained in the nucleus for assembly of viral particles, while additional M protein could be exported for cell-to-cell movement.

In plants, nucleocytoplasmic partitioning pathways can be loosely grouped into four categories, namely 1) phosphorylation or conformational change masking or exposing an NLS or NES; 2) heterocomplex formation resulting in 'piggybacking' into the nucleus; 3) cytoplasmic or nuclear retention due to protein:protein interactions; and 4) cytoplasmic or nuclear retention due to membrane association (Meier and Sommers, 2011). Further complicating identification of import pathways is the fact that none of these groups are mutually exclusive, as we have

demonstrated in this study. This emphasizes the importance of characterizing NLSs and import pathways in order to more accurately predict the sub-cellular localization of proteins.

TABLE 4.1. Predicted nuclear localization signals in matrix proteins of nucleorhabdoviruses.

Only the PYDV signal has been mapped physically, others have been predicted using *in silico* methods. The numbers indicate the amino acid residues in the full-length proteins that mark the NLSs. MFSV, maize fine streak virus; SYNV, sonchus yellow net virus; RYSV, rice yellow stunt virus; MMV, maize mosaic virus; IMMV, Iranian maize mosaic virus; TaroVCV, taro vein chlorosis virus.

Virus	Genbank Accession	Type	Predicted NLS
MFSV	NC_005974.1	Bipartite	¹⁹⁵ KKEDKA EKATTEKRKRQ ²¹¹
SYNV	KF264454.1	Dual monopartite	²³⁰ RKRK ²³³ , ²⁶⁷ RKHR ²⁷⁰
RYSV	NC_003746.1	none	n/a
MMV	NC_005975.1	none	n/a
IMMV	NC_011542.1	none	n/a
TaroVCV	NC_006942.1	none	n/a
PYDV	NC_016136.1	Bipartite	²¹³ KKTVSDPLKNLLKRKSD ²²⁹

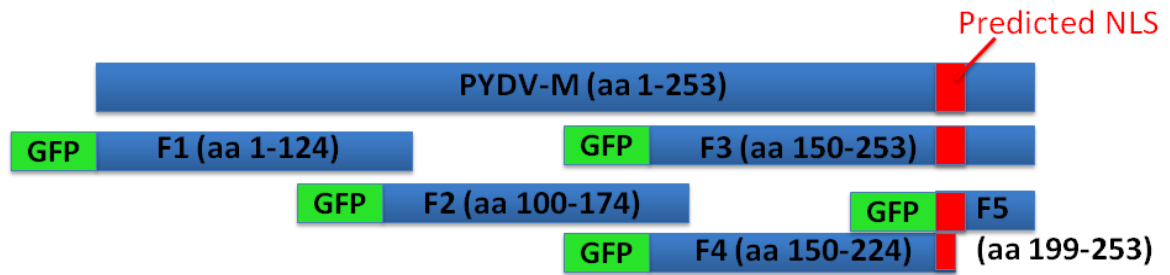


FIGURE 4.1. Size and position of deletion fragments of PYDV-M.

Five overlapping fragments were created from the 253 amino acid PYDV-P protein.

F1 is the N-terminal 124 amino acids of PYDV-M, and is expected to have a molecular mass of 41 kDa. F2 spans amino acids 100-174 of PYDV-M, and has a predicted mass of 35 kDa. F3, which corresponds to amino acids 150-253 of PYDV-M, and the mass is predicted to be 38 kDa. F4 and F5 are sub-fragments of F3, and correspond to amino acids 150-224 (F4) and 199-253 (F5) of PYDV-M. The predicted NLS is indicated as a red band.

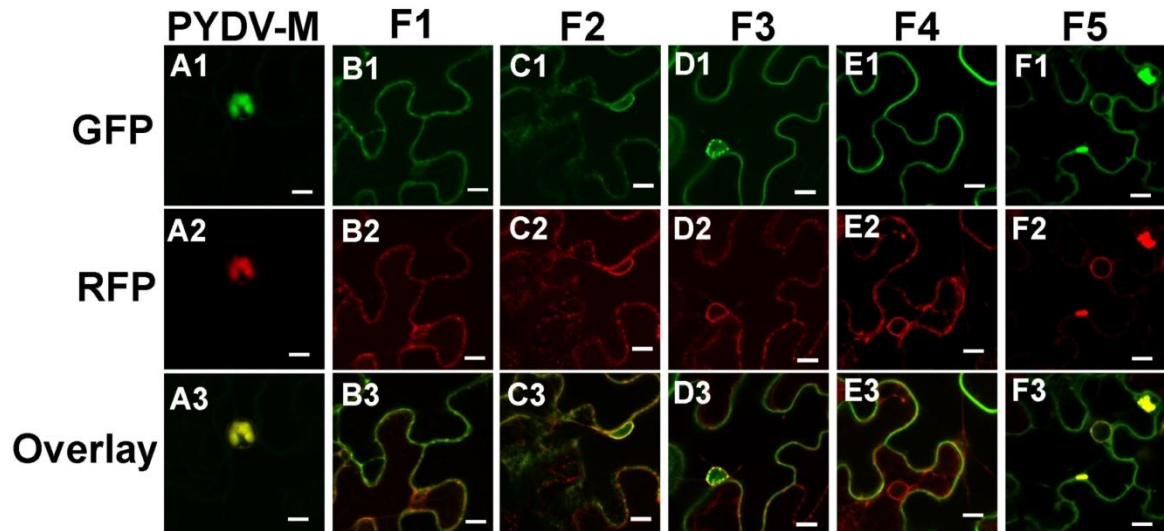


FIGURE 4.2. Confocal micrographs showing localization of GFP:protein fusions expressed transiently in transgenic *N. benthamiana* plants expressing a RFP-ER marker.

Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP-ER marker, and the resultant overlay, respectively. **(A1-3)** Localization of GFP:M fusion **(B1-3)** Amino acids 1-124 of PYDV-M fused to GFP. **(C1-3)** Amino acids 100-174 of PYDV-M fused to GFP. **(D1-3)** Amino acids 150-253 of PYDV-M fused to GFP. **(E1-3)** Amino acids 150-224 of PYDV-M fused to GFP. **(F1-3)** Amino acids 199-253 of PYDV-M fused to GFP. Scale bar = 10 μ m.

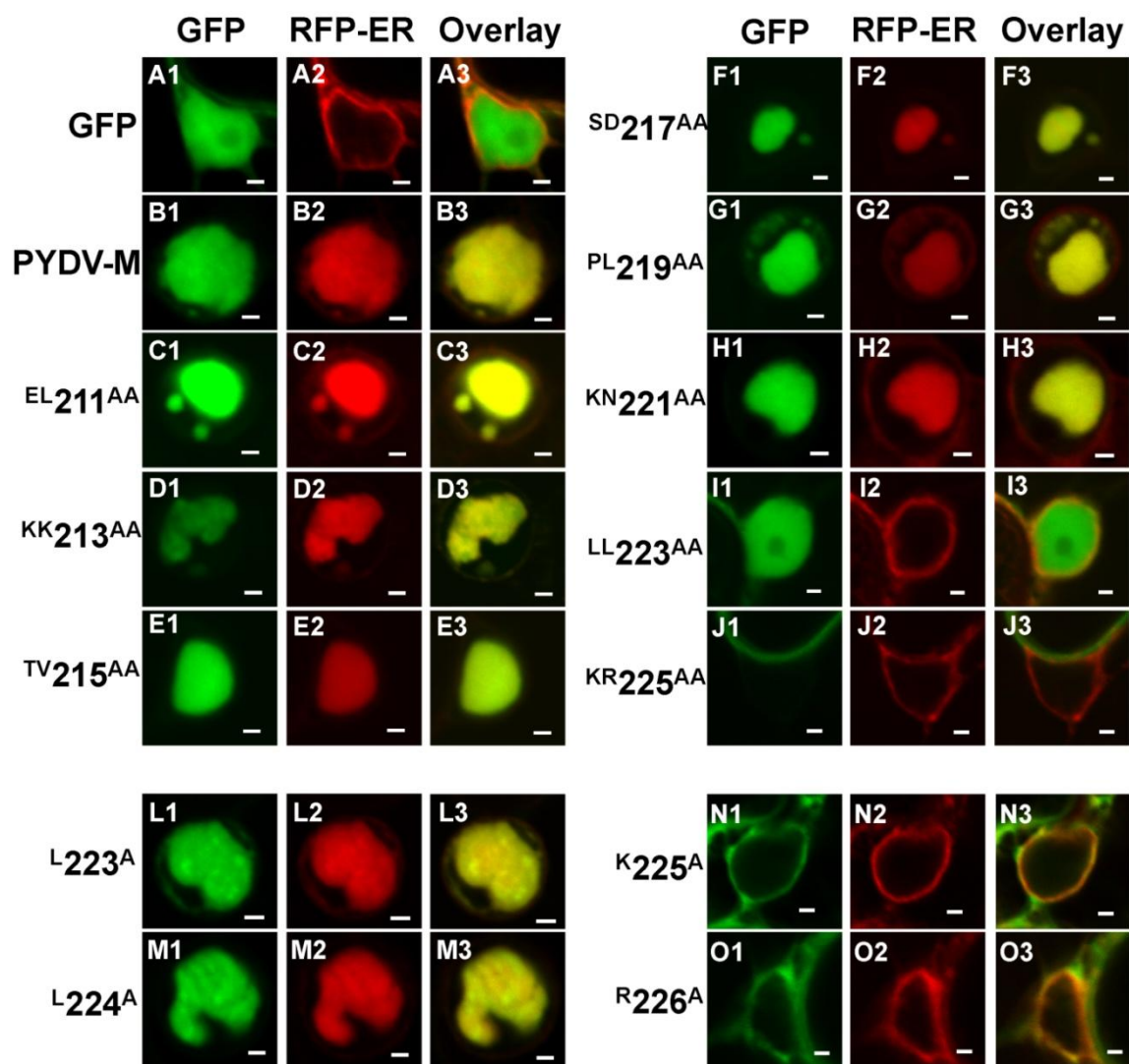


FIGURE 4.3. Single plane confocal micrographs of PYDV-M and site-directed mutants fused to GFP, expressed in transgenic *N. benthamiana* expressing an RFP-ER marker.

Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP-ER marker, and the resultant overlay, respectively. **(A1-3)** GFP only. **(B1-3)** GFP:PYDV-M. **(E-J)** PYDV-M site-directed double mutants. Numbers indicate the amino acid residue in the full-length M protein where a two-residue change to alanine was introduced. **(L-O)** PYDV-M site-directed single mutants. Numbers indicate the amino acid residue in the full-length M protein where a single residue change to alanine was introduced. Scale bar = 2 μ m.

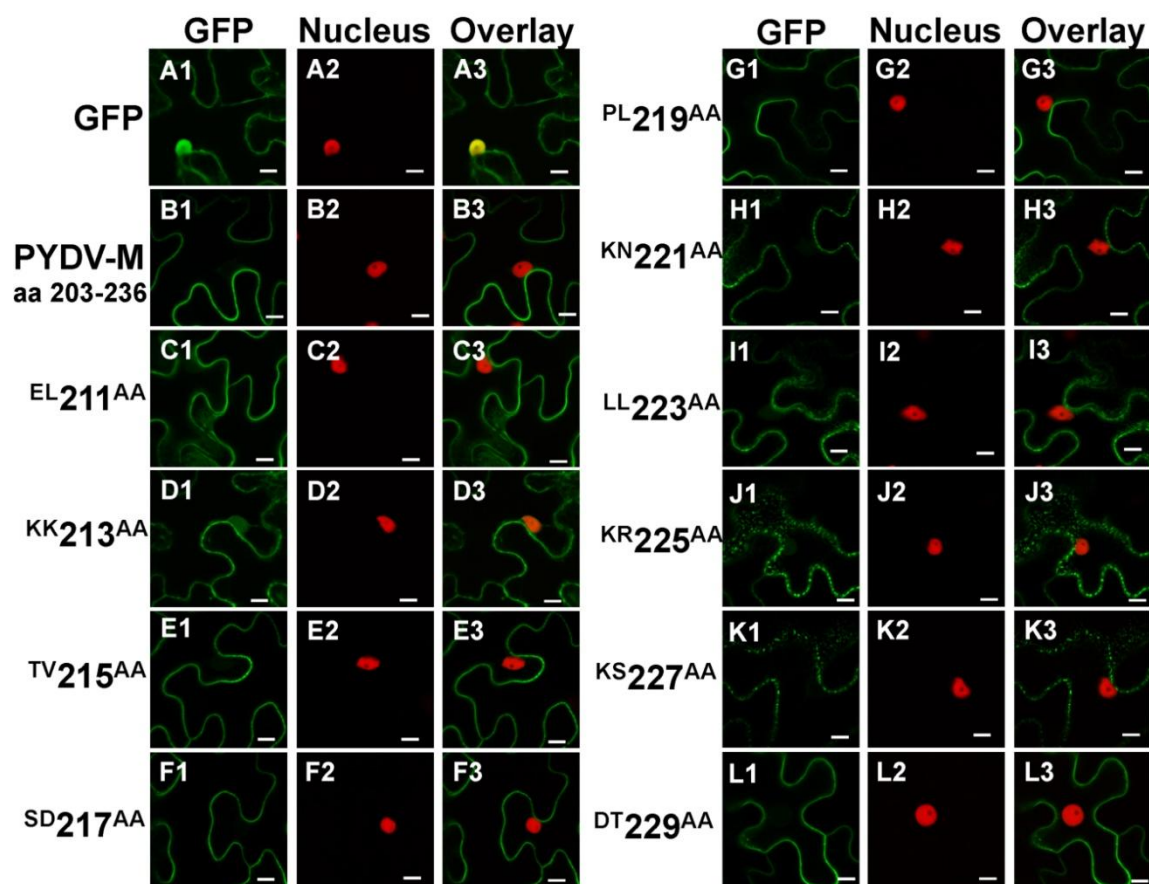


FIGURE 4.4. Single-plane confocal micrographs of amino acids 203-236 of PYDV-M and site-directed mutants expressed in transgenic *N. benthamiana* expressing RFP fused to a nuclear marker, histone 2B.

Shown in panels 1, 2, and 3 are micrographs of GFP-fusions, RFP-nuclei, and the resultant overlay, respectively. **(A1-3)** GFP only. **(B1-3)** Amino acids 203-236 of PYDV-M fused to GFP. **(C-L)** Site-directed double mutants of the 203-236 aa fragment of PYDV-M. Numbers indicate the amino acid residue in the fragment where a two-residue change to alanine was introduced. Scale bar = 10 μ m.

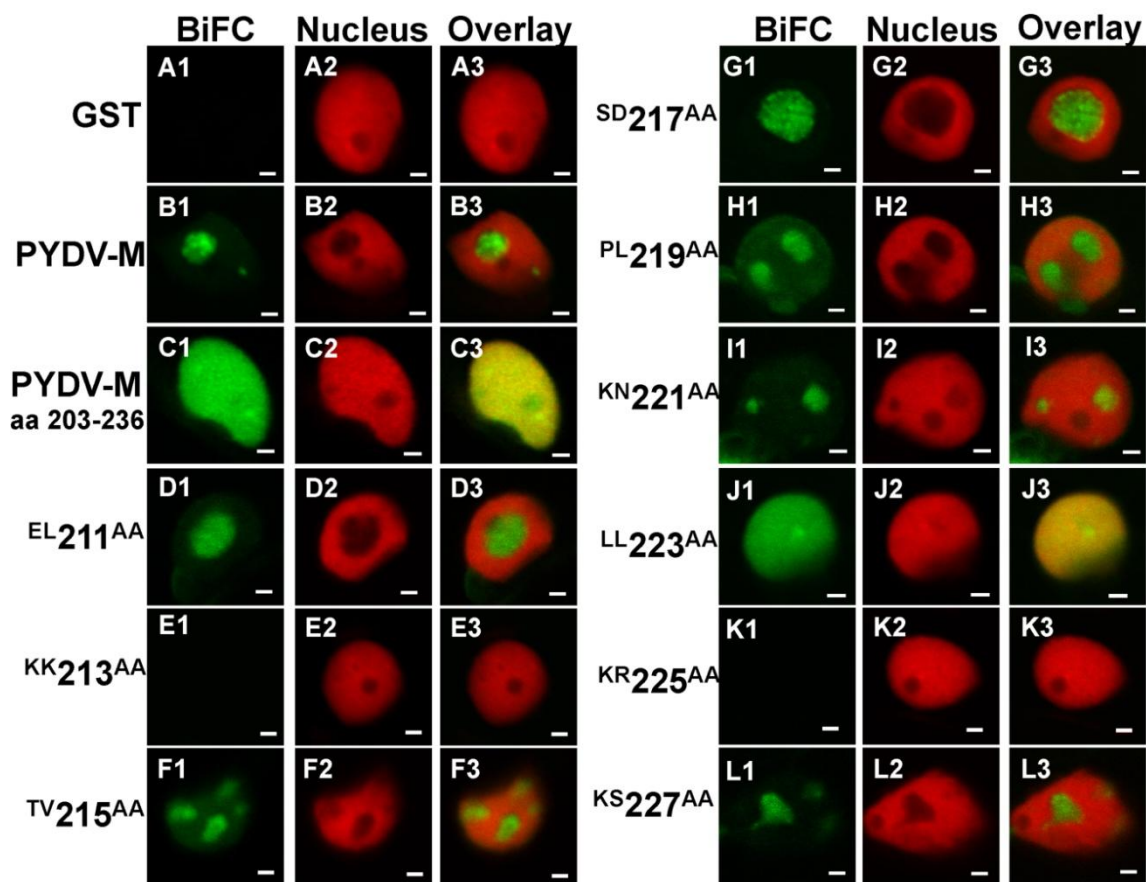


FIGURE 4.5. Confocal micrographs showing results of BiFC assay to determine interaction between importin- α and PYDV-M, PYDV-M NLS, and PYDV-M mutants.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing fluorescent nuclear marker protein fusions. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against importin- α isoforms expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC of GST and importin- α . **(B1-3)** BiFC between importin- α and PYDV-M. **(C1-3)** BiFC between the predicted NLS of PYDV-M (aa 203-236) and importin- α . **(D-L)** BiFC between importin- α and double mutants of PYDV-M, described in Figure 4.3. Scale bar = 2 μ m.

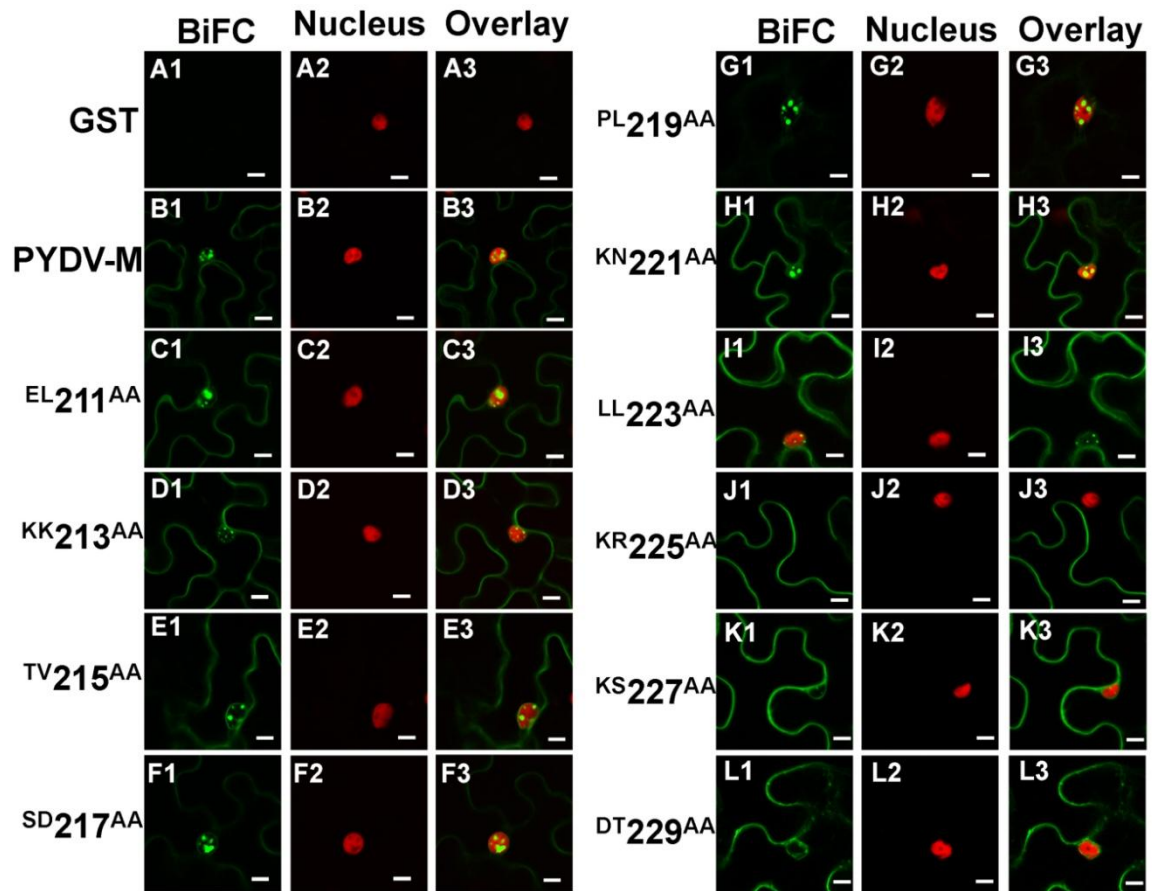


FIGURE 4.6. Confocal micrographs showing PYDV-M mutant protein interactions with wild-type PYDV-M determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against wild-type PYDV-M expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC between GST and PYDV-M. **(B1-3)** BiFC self-interaction of PYDV-M. **(C-L)** BiFC between PYDV-M and double mutants of PYDV-M. Scale bar = 10 μ m.

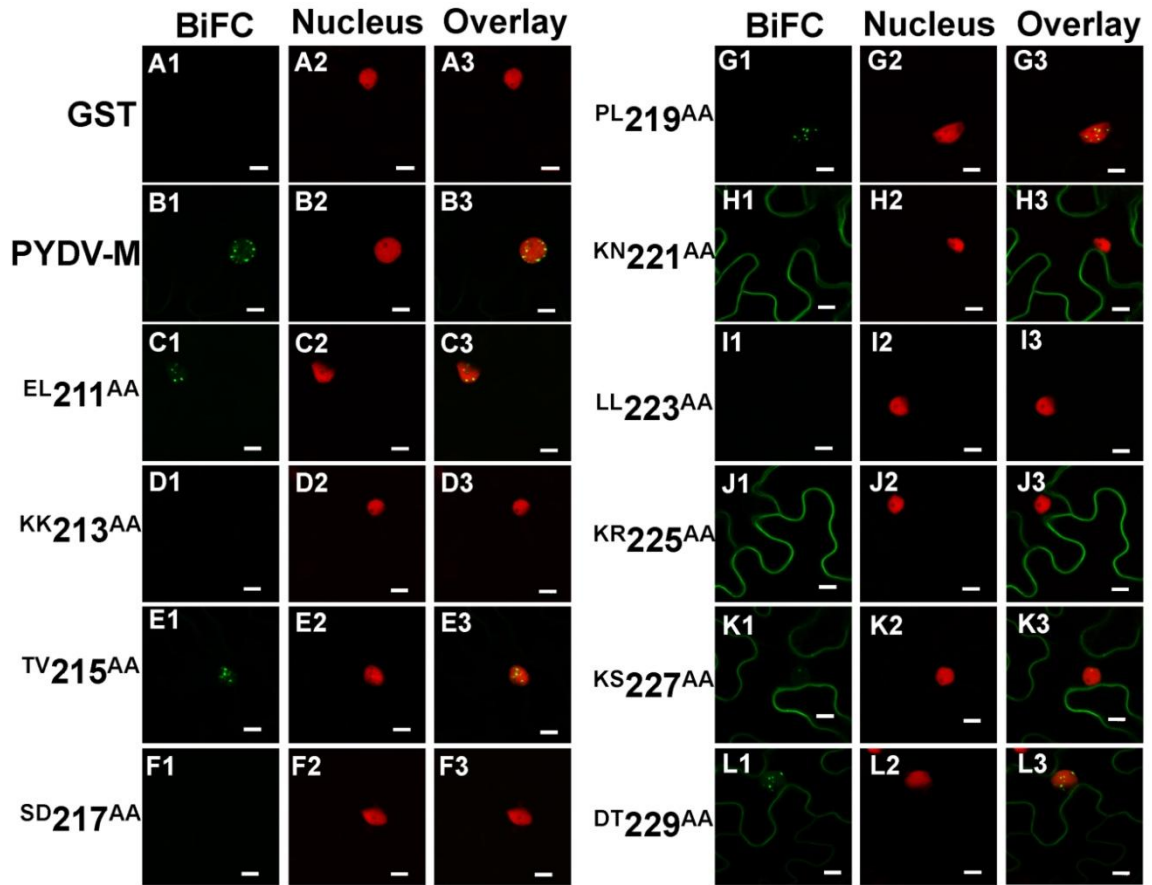


FIGURE 4.7. Confocal micrographs showing PYDV-M mutant protein interactions with PYDV-Y determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-Y expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC of GST and PYDV-Y. **(B1-3)** BiFC between PYDV-M and PYDV-Y. **(C-L)** BiFC between PYDV-Y and double mutants of PYDV-M. Scale bar = 10 μ m.

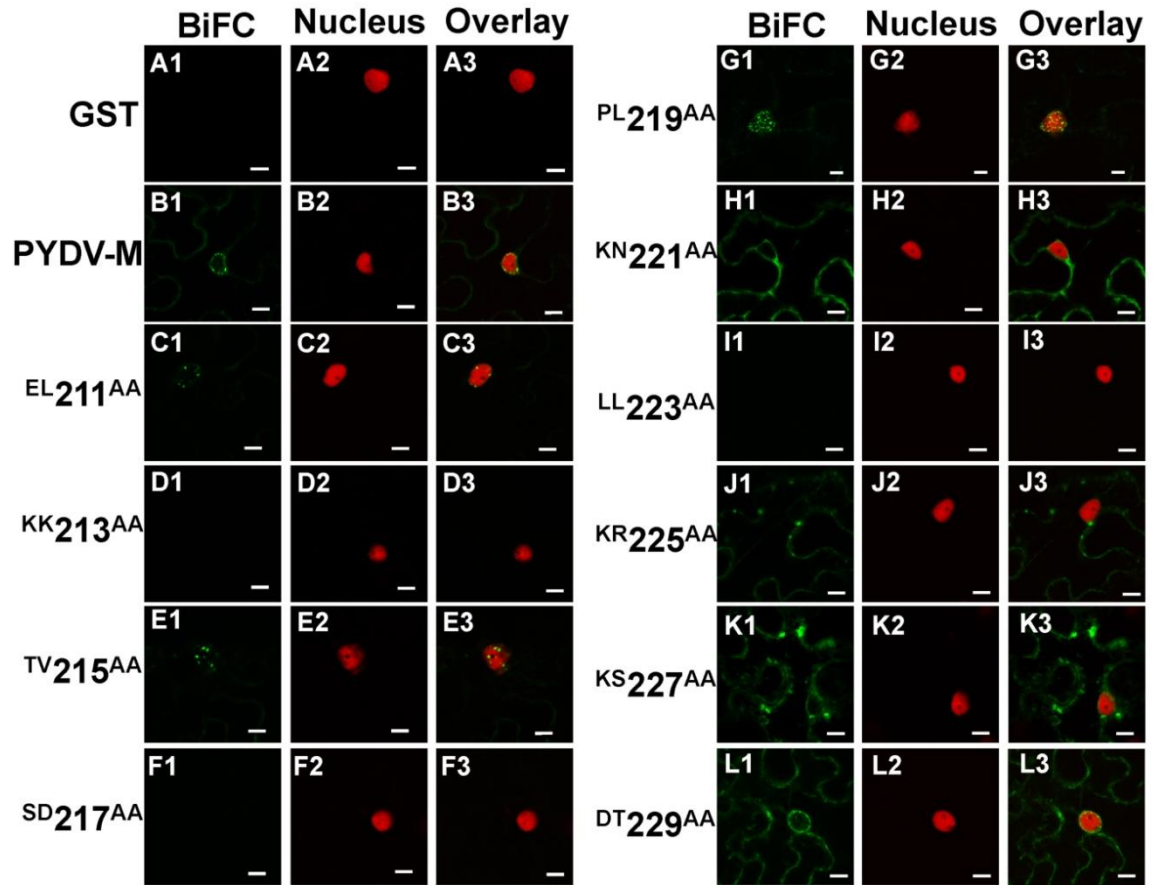


FIGURE 4.8. Confocal micrographs showing PYDV-M mutant protein interactions with PYDV-G determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-G expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC between GST and PYDV-G. **(B1-3)** BiFC between PYDV-M and PYDV-G. **(C-L)** BiFC between PYDV-G and double mutants of PYDV-M. Scale bar = 10 μ m.

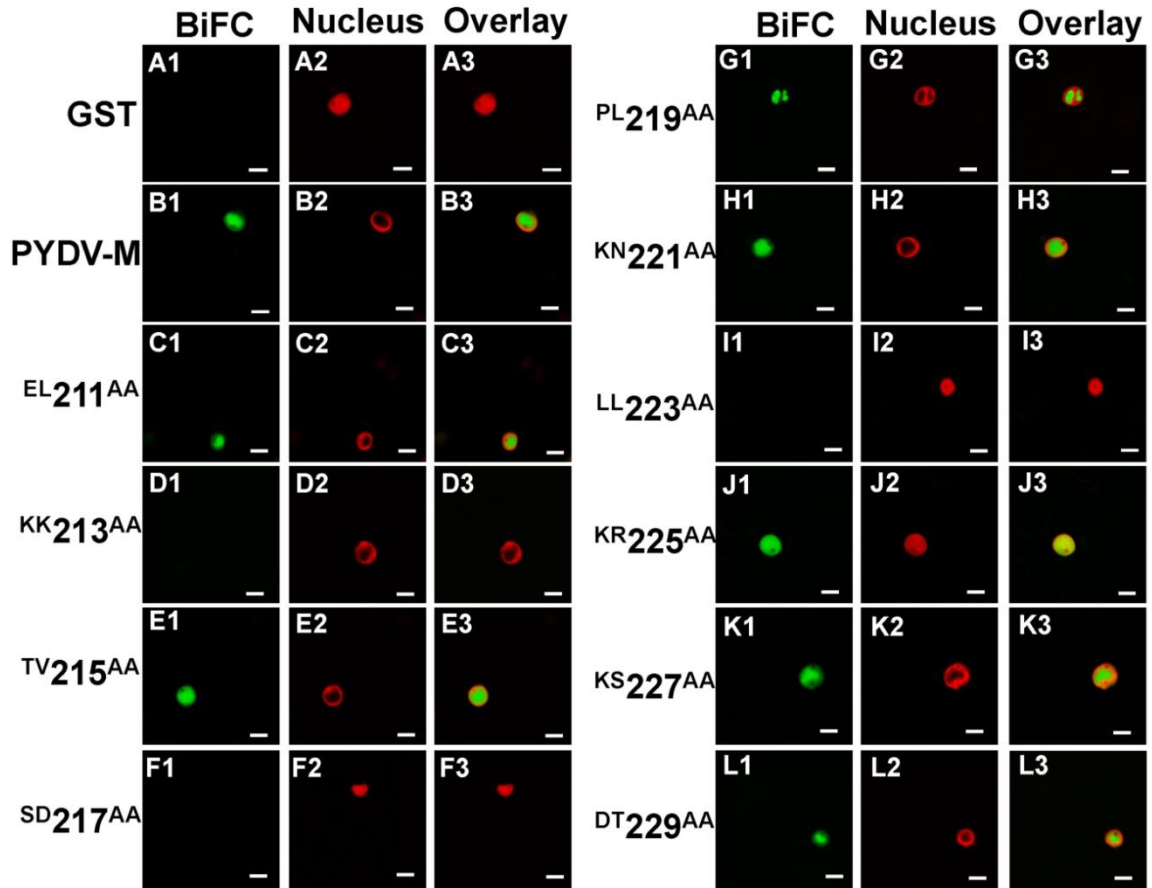


FIGURE 4.9. Confocal micrographs showing PYDV-M mutant protein interactions with PYDV-N determined by BiFC.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing a fluorescent nuclear marker protein fusion. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against PYDV-N expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC between GST and PYDV-N. **(B1-3)** BiFC between PYDV-M and PYDV-N. **(C-L)** BiFC between PYDV-N and double mutants of PYDV-M. Scale bar = 10 μ m.

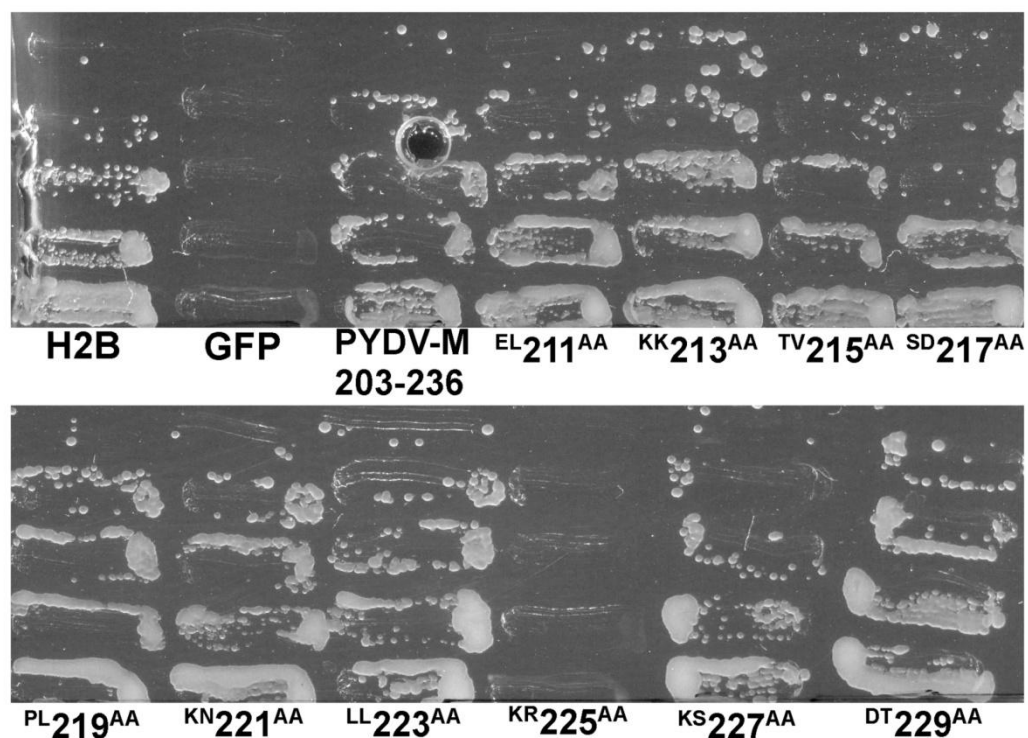


FIGURE 4.10. Yeast-based assay for identification of proteins containing a functional NLS.

Positive [histone 2b (H2B)] and negative (GFP) control proteins, along with amino acids 203-236 of PYDV-M and mutants thereof, were expressed from pNIA-DEST in yeast strain L40. Only proteins containing a functional NLS are able to facilitate yeast growth on media lacking histidine, and were scored as positive in this assay.

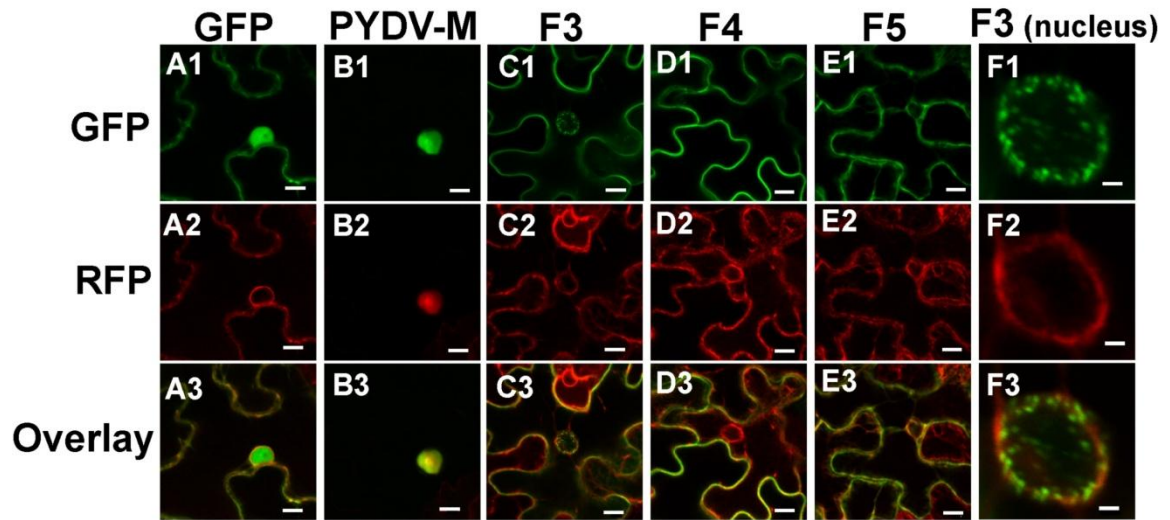


FIGURE 4.11. Confocal micrographs showing localization of GFP:protein fusions containing the ^{LL223AA} mutation expressed transiently in transgenic *N. benthamiana* plants expressing a RFP-ER marker.

Shown in panels 1, 2, and 3 are micrographs of GFP (fusions), RFP-ER, and the resultant overlay, respectively. **(A1-3)** Localization of GFP only. **(B1-3)** Localization of GFP:M fusion. **(C1-3)** Amino acids 150-253 of PYDV-M containing the ^{LL223AA} mutation. **(D1-3)** Amino acids 150-224 of PYDV-M containing the ^{LL223AA} mutation. **(E1-3)** Amino acids 199-253 of PYDV-M containing the ^{LL223AA} mutation. **(F1-3)** Nuclear view of **(C1-3)**. Scale bar = 10 μ m (cell) or 2 μ m (nucleus).

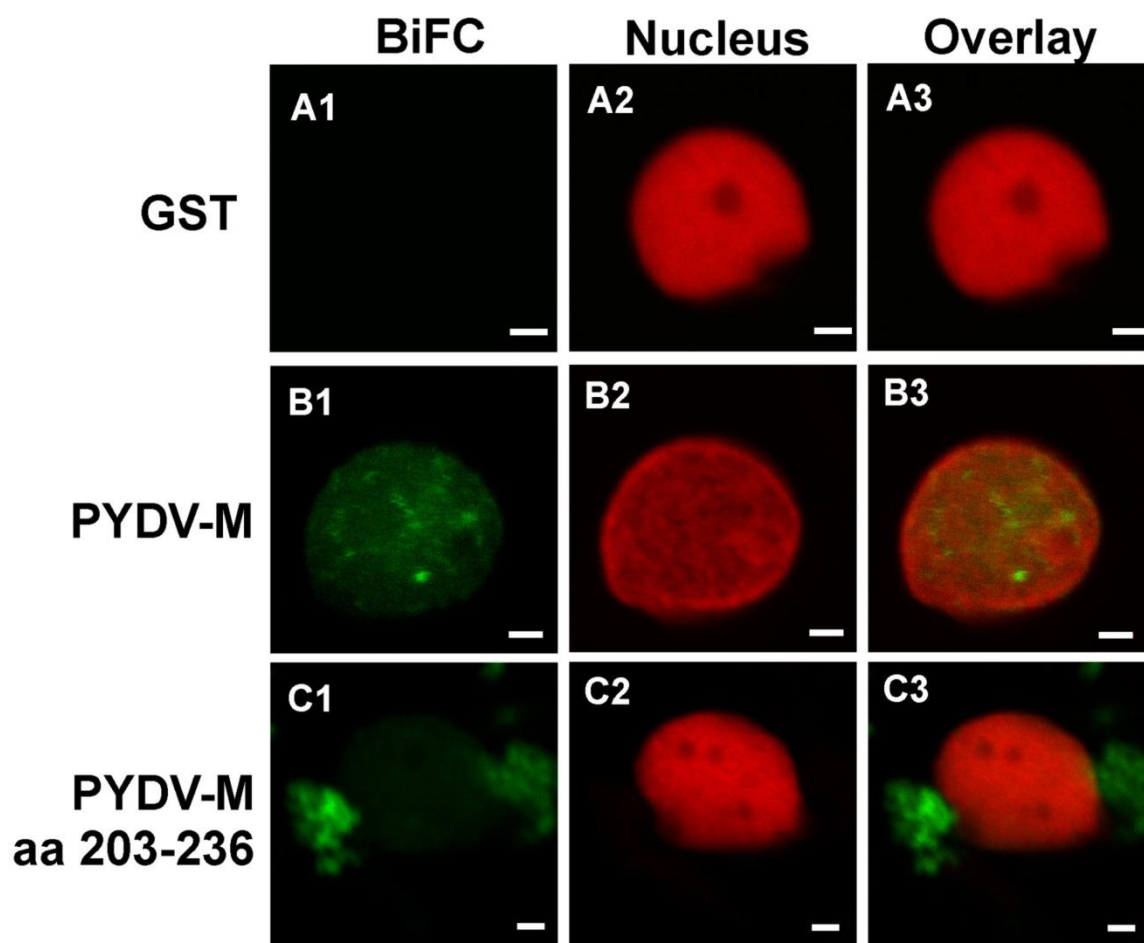


FIGURE 4.12. Confocal micrographs showing results of BiFC assay to determine interaction between XPO1 and PYDV-M or PYDV-M NLS.

Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing fluorescent nuclear marker protein fusions. Panels 1, 2, and 3 show micrographs of YFP (BiFC), fluorescent nuclear marker, and the resultant overlay, respectively. All interactions were tested against *A. thaliana* XPO1 expressed as a fusion to the carboxy-terminal of YFP. **(A1-3)** BiFC between GST and XPO1. **(B1-3)** BiFC between XPO1 and PYDV-M. **(C1-3)** BiFC between the predicted NLS of PYDV-M (aa 203-236) and XPO1. Scale bar = 2 μ m.

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